University of Alberta

Characterization of the Ecdysteroid Receptor in the Salivary Gland of the Ixodid Tick, *Amblyomma hebraeum* Koch

by

Helen Mao

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of DOCTOR OF PHILOSOPHY

in

Physiology and Cell Biology

Department of Biological Sciences

Edmonton, Alberta

Fall, 1998

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: Characterization of the Ecdysteroid Receptor in the Salivary Gland of the Ixodid Tick, *Amblyomma hebraeum* Koch, submitted by Helen Mao in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences (Physiology and Cell Biology).

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Abstract

The salivary glands of *Amblyomma hebraeum* Koch (Acari: Ixodidae) play important roles in tick feeding. Following engorgement, salivary glands degenerate, a process initiated by an ecdysteroid hormone. I have detected an ecdysteroid receptor (EcR) in salivary gland tissue extracts. The EcR binds to [^{3}H]ponasterone A (Pon A) with high affinity (Kd = 1 nM) and limited capacity. Displacement of Pon A from the EcR by ecdysteroids has the following order of affinity: Pon A > muristerone A > makisterone A > 20-hydroxyecdysone > mesylinokosterone > ecdysone. Vertebrate steroids could displace little Pon A binding.

The EcR shifted the [^{32}P]hsp27 EcRE band on a gel mobility shift assay. A number of ecdysteroids enhanced the binding of EcR to hsp27 EcRE with various potencies, which agreed with their binding affinities, whereas vertebrate steroids had no such effect. Using monoclonal antibodies against *Drosophila* EcR (AG10.2) and against *ultraspiracle* protein (USP; AB11), three EcR bands (90.5, 87.3 and 84 kDa) and four major USP bands (52.6, 50.3, 47.1 and 43.7 kDa) were detected in western blot. In addition, AB11 supershifted the tick EcR-hsp27 EcRE band on a gel mobility shift assay, suggesting that the tick EcR heterodimerized with a USP-like protein for DNA binding. Furthermore, selective mutations to the 15 basepair palindrome of hsp27 EcRE at positions -5, or +2, or adding a basepair to the spacer, resulted in considerably reduced affinity to the tick EcR/USP. These results suggest that tick EcR/USP binds to hsp27 EcRE sequence specifically and tick EcRE might be highly-conserved.

I examined profiles of hemolymph ecdysteroid titer (by RIA) and the EcR/USP (ligand-binding assay, western blot) in the salivary gland throughout the feeding period and 6 days post-engorgement. A distinguish hemolymph ecdysteroid peak was detected during the rapid phase of feeding. EcR was induced after the onset of feeding, sustained during feeding and declined during post engorgement period. After ticks exceeded 100 mg, a discrete DNA-binding band was shown by gel mobility shift assay. Maximal DNA-binding

coincided with the hemolymph ecdysteroid peak during the rapid phase of feeding, which might be the signal to trigger salivary gland degeneration. From these results, I proposed a model to explain the mechanism of ecdysteroid action on salivary gland degeneration.

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Abbreviations Used in this Thesis

20E	20-hydroxyecdysone
Ab	antibody
BCIP	5-bromo-4-chloro-3-indoylphosphate p-toluidine
Chl	cholesterol
CT-DNA	calf thymus DNA
DBD	DNA-binding domain
E	ecdysone
EcR	ecdysteroid receptor
EcRE	ecdysone response element
ERE	estrogen response element
Est	ß-estradiol
GRE	glucocorticoid response element
HBD	hormone-binding domain
hsp	heat shock protein
JH	juvenile hormone
JHR	juvenile hormone receptor
mAb	monoclonal antibody
Mak A	makisterone A
MIS	mesylinokosterone
Mur A	muristerone A
NBT	p-nitro blue tetrazolium chloride
PgR	progesterone receptor
Pon A	ponasterone A
RAR	retinoid acid receptor
RXR	9-cis retinoid acid receptor
TR	thyroid hormone receptor

TRE	thyroid hormone response element
Tst	testersterone
USP	*ultraspiracle* protein
VDR	vitamin D_3 receptor

Chapter 1

General Introduction

Ticks: General Background, Feeding and Reproduction

Ticks are obligate ectoparasites that infest every class of terrestrial vertebrates. They are classified into three families: (i) Ixodidae (hard ticks, ~650 species), (ii) Argasidae (soft ticks, 169 species) and (iii) Nuttaliellidae (a single species, figure 1.1). Damage caused by ticks to their hosts results directly from exsanguination and indirectly from transmission of pathogens and toxins. The pathogenic organisms transmitted include fungi, viruses, rickettsiae, spirochetes, bacteria, protozoa, and even filarial nematodes (Sonenshine, 1991). The African tick, *Amblyomma hebraeum* Koch — is noteworthy for transmitting *Cowdria ruminantium*, a rickettsia which causes heartwater in ruminants. It is one of the most important tick-borne diseases of cattle in Africa (Provost and Bezuidenhout, 1987). *A. hebraeum* also transmits *Rickettsia conori*, the etiologic agent of 'tick-bite fever' in humans (Kelly *et al*, 1994). The rest of this review concentrates on the Ixodidae.

The blood ingested by a female ixodid tick amounts to several hundred times its unfed weight. Much of the fluid portion of the blood meal is excreted back to the host during the feeding period; thus at full engorgement, the fed-to-unfed weight ratio is about 100 for many ixodid species. Male ixodid ticks ingest much less blood, the fed-to-unfed weight ratio usually being less than 2.

In ixodid female ticks, the feeding cycle is divided into three phases: (i) The first 24-36 hours following attachment is called 'the preparatory phase', during which ticks establish the feeding lesion. A cement-like substance secreted from the salivary glands anchors the mouthparts to the insertion site. (ii) The 'slow phase of feeding' usually lasts 7-8 days. It is also referred as the "growth phase" by Araman (1979), because a variety of tissues and organs (cuticle, salivary gland, gut, ovary etc.) undergo considerable growth and differentiation during this time. In most species, mating takes place at this stage as

well. The female increases her body weight 10-fold during the slow phase of feeding. (iii) The last phase lasts only 12-24 h and is thus called the 'rapid phase of feeding'. The female increases her weight a further 10-fold at full engorgement. At the transition between the slow and rapid phases of feeding there is a marked change in a number of behaviors (reviewed by Kaufman and Lomas, 1996). Because those behavioral changes are linked more specifically to achieved weight rather than to duration of feeding, it has been called the 'critical weight' (Harris and Kaufman, 1984; Lindsay and Kaufman, 1988; Kaufman and Lomas, 1996).

Ixodid ticks lay only a single batch of eggs following engorgement. The size of the egg mass is directly correlated with the engorged weight. In larger species, such as *A. hebraeum*, it can approach 20,000 eggs. This represents about 50% of the engorged weight of the tick (Logan *et al.*, 1989; Kaufman *et al*, 1986).

Structure and Function of the Salivary Gland

The salivary glands are a pair of elongated, glandular masses located laterally in the body cavity of the tick. There are three types of acini in the salivary gland of females (figure 1.2) and four in males. The gland is innervated by a branch of a pedipalpal nerve (Megaw, 1977) which ramifies and enters the base of all acini and terminates in synapses on the cells (Fawcett *et al.*, 1986). Secretory function of the salivary gland is under neural control, with dopamine being the neurotransmitter (Megaw and Robertson, 1974; Kaufman, 1977).

Type I acini attach directly to the anterior portion of the main duct and are composed of four 'agranular' cell types (central, pyramidal, neck and constrictor cells; figure 1.2). It is believed that acini I are involved in actively absorbing atmospheric water vapour. They do this by secreting a hygroscopic saliva droplet on the mouthparts during the non-feeding period; following sorption of the water, the droplet is swallowed (Knülle and Rudolph, 1982; Needham and Teel, 1986).

Type II acini consist mainly of ***a***, ***b*** and **c** granular cells, and are located along the secondary branches of the main duct (figure 1.2). Acini III, located more posteriorly to the secondary duct, consist of ***d***, ***e*** and ***f*** granular cells. All of the granular cells are separated by abluminal (AbIC) and adluminal interstitial cells (AdIC). The ***a***, ***d*** and ***e*** cells contain lipoprotein-positive staining granules, which are depleted during the preparatory phase of feeding. It has been speculated, therefore, that they secrete the cement that secures the mouthparts of the tick to the host's skin (Binnington, 1978; Kemp et al., 1982; Gill and Walker, 1987). The staining properties of the c cell granules suggest that they contain an anti-clotting proteoglycan substance (Geczy et al., 1971; Walker and Fletcher, 1987). During the slow phase of feeding, the ***f*** cells develop extensive baso-lateral membrane infoldings which interdigitate with the adjacent AbICs to form an 'extracellular labyrinth' (Binnington, 1978; Fawcett et al., 1981). This labyrinth is believed to be the site of fluid transport across the epithelium.

Salivary Gland Development

The salivary glands undergo dramatic growth and differentiation during the slow phase of feeding (Till, 1961). A number of biochemical studies show that gene activation (Oak et al., 1991), *de novo* mRNA synthesis (Shelby et al., 1987) and protein synthesis (Neitz and Gothe, 1986; McSwain et al., 1982) occur during this phase of the feeding cycle. Feeding and mating are two major events which appear to induce these biochemical changes. Coons and Kaufman (1988) found that development of the extracellular labyrinth can be induced by implanting the salivary glands of unfed ticks into the hemocoel of feeding ticks, which suggests that a hemolymph-borne factor is responsible for salivary gland development. The identity of this factor remains unknown.

Salivary Gland Degeneration

The salivary glands of engorged females degenerate within 4 days post-detachment. The autolytic process is characterized by the appearance of autophagic vacuoles (Harris and Kaufman, 1981) and by the loss of fluid secretory capacity (Harris and Kaufman, 1984). Harris and Kaufman (1981) demonstrated that transplantation of salivary glands from small partially fed ticks (glands which do not degenerate) into the hemocoel of engorged ticks induced the development of autophagic vacuoles in the transplanted salivary glands. A subsequent study (Harris and Kaufman, 1985) demonstrated that: (i) salivary gland degeneration can be induced *in vitro* by exposing the glands from small partially-fed ticks to physiological concentrations of 20-hydroxyecdysone (20E); (ii) degeneration can also be induced *in vivo* by infusing small partially-fed females with graded doses of 20E; finally, (iii) Kaufman (1991) demonstrated a dramatic increase of ecdysteroid titer in the hemolymph over the first week post-engorgement. Moreover, Lindsay and Kaufman (1988) showed that vertebrate steroids (cortisol, ß-estradiol, progesterone, testosterone) did not cause salivary gland degeneration *in vitro*, indicating that the degeneration effect is ecdysteroid-specific. In fact, vertebrate steroids caused a significant increase in fluid secretory competence of the salivary gland in *A. americanum* (Lindsay and Kaufman, 1988); the mechanism behind this effect is not yet known.

Apoptosis, or programmed cell death, is an orderly and genetically controlled form of cell death. It differs from necrosis in that cellular shrinkage and chromatin condensation occur, followed by fragmentation of nuclear components. Membrane-bound vesicles are formed in the cytoplasm and are cleared by phagocytosis without damage to adjacent tissue. The following evidence indicates that salivary gland degeneration is an apoptotic process: (i) it is a hormonally controlled process; (ii) autophagic vacuoles are present in the degenerating tissue; (iii) fragmentation of nuclear components have been observed in the degenerating tissue (unpublished observations); and (iv) transglutaminase activity, a marker

enzyme characterized in apoptotic tissue, has been detected and its level increases in the salivary gland tissue over a 2-day period post-engorgement (unpublished results).

Structure and Biological Activity of Ecdysteroids in Insects

Ecdysteroids† are a group of polyhydroxylated polar steroids (figure 1.3). Polyhydroxylation renders ecdysteroids far more water-soluble than most vertebrate steroids. Structural features of the ecdysteroids used in this thesis are summarized in figure 1.3.

Horn and Bergamasco (1985) have reviewed the structure-activity relationships of a variety of ecdysteroids. Basically, the critical structural features for ecdysteroid activity are those that distinguish them from vertebrate steroids (figure 1.4): (i) a *cis*-fused A/B ring junction, *i.e.*, a 'chair' structure, (ii) a 6-keto-7-ene grouping and (iii) a full sterol side chain. Some modifications on the side chain, *e.g.*, 2ß-, 3ß-, 20R-hydroxy, groups enhance bioactivity, but a 25-hydroxy group diminishes activity. Structures of the ecdysteroids and vertebrate steroids used in this thesis are shown in figure 1.5 and figure 1.6 respectively.

The Ecdysteroid Receptor (EcR)

EcR belongs to the 'nuclear receptor' superfamily with over 150 known members (reviewed by Mangelsdorf *et al.*, 1995). They are a group of ligand-activated transcription factors, which include receptors for vertebrate steroid hormones, vitamin D_3 (VDR), thyroid hormone (TR), retinoic acid (RAR), and some 'orphan receptors' (receptors for which the natural ligands remain unknown; Evans 1988). Members in this superfamily all contain the highly conserved C_2C_2 zinc finger structure (figure 1.7) in the DNA-binding

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† In this thesis, ecdysteroid is a generic name for a group of structurally-related chemicals. Ecdysone, in most cases, refers to the specific compound (see figure 1.5). However, it is also used in a generic sense in the phrase 'ecdysone response element'.

domain (DBD), which distinguishes them from other DNA-binding proteins. The characteristic functions for a nuclear receptor are: (i) recognition and binding to a specific ligand, (ii) recognition and binding to a specific DNA sequence (hormone response element), and (iii) activation or repression of transcription. The functions of EcR are carried out by five modular regions (figure 1.8):

(1) The N-terminal A/B region is one of the most variable parts among the five regions in terms of length and sequence identity among EcRs (reviewed by Fujiwara *et al.*, 1995). Functions such as transcriptional activation or repression has been proposed for this region (Cherbas and Cherbas, 1996).

(2) The DBD (C region) is the most conserved region among the superfamily members. Up to 95-100% identity within a fixed number of 66 amino acids are found among several cloned EcRs (summarized by Fujiwara *et al.*, 1995, figure 1.8) and USPs (summarized by Jindra *et al.*, 1997, figure 1.9). The C_2C_2 zinc-finger motif is constituted by 8 cysteine residues coordinated tetrahedrally with 2 zinc atoms (figure 1.7). Within the finger region, a P box sequence is important for recognition of a hormone response element (Danielson *et al.*, 1989; Mader *et al.*, 1989). TR, VDR, RAR and EcR of the same subfamily share the same P box sequence (EGckG), which also binds to the same sequence of response element half-site (AGGTCA).

(3) The hinge region (D region) is also quite variable, and contains a putative nuclear localization signaling sequence. The sequence directs the receptor to the nucleus after synthesis, even in the absence of the hormone (LaCasse *et al.*, 1993; Koelle *et al.*, 1991). The region also provides a flexible hinge that allows the receptor to fold (Tsai and O'Malley, 1994).

(4) The HBD (E region) is less conserved than DBD and is responsible for ligand binding and dimerization. The ligand binding sites are variable, and scattered residues in a large region are involved (Nagaya and Jameson, 1993a). A pocket formed by hydrophobic residues in HBD is speculated to hold the steroid ligand (Ojasoo *et al.*, 1990). Affinity

labeling studies have shown that some residues in that pocket are responsible for ligand-receptor interaction, among which cysteine is the most frequent residue at the contact points (Simons *et al.*, 1987; Harlow *et al.*, 1989). HBD also contains 9 highly conserved heptad repeats of hydrophobic residues (Forman and Samuels, 1990), which is characteristic of a "leucine zipper" structure in protein-protein interaction (Sassone-Corsi, 1992). The 9 heptad repeats are essential for dimerization (Nagaya and Jameson, 1993a, b).

(5) The F-region is the least conserved region among the cloned EcR (figure 1.8). The function of this region is unknown.

ultraspiracle Protein (USP)

When Perrimon *et al* (1985) did genetic tests on zygotic lethal genes on the X chromosome of *Drosophila*, they named one of those lethal genes *ultraspiracle*. The *usp*/Y males usually died between the first and second larval instar. The fly frequently completed the molt but retained the spiracle structure of the first instar larva, and so possessed an extra set of spiracles — thus 'ultraspiracle'. USP, the *usp* gene product, shares 86% and 49% sequence identity to mammalian homolog RXR (retinoic X receptor) in the DBD and LBD regions respectively. Similarly, as RXR dimerizes with TR/VDR/RAR, USP heterodimerizes with EcR to form a functional receptor (Yao *et al.*, 1992; 1993; Swevers *et al.*, 1996). Among the cloned USPs of *Drosophila* (Khoury Christianson *et al.*, 1992), *Bombyx mori* (Tzertzinis *et al.*, 1994) and *Manduca sexta* (Jindra *et al.*, 1997), a high degree of sequence identity occurs in the DBD region (figure 1.9), which also contains the C_2C_2 zinc finger motif. One copy of *usp* mRNA in *Drosophila* is expressed widely with varied amounts at all developmental stages (Henrich *et al.*, 1994). Two copies of *usp* mRNA are expressed alternatively in *Manduca*, which contributes to the constitutive expression of the total *usp* mRNA (Asahina *et al.*, 1997).

JH seems to be a natural ligand of USP (Jones and Sharp, 1997). Upon binding to JHIII, USP undergoes a conformational change and induces transcription *in vitro* (Jones

and Sharp, 1997). These results shed light, at the receptor level, on the interactions between ecdysteroid hormone and JH that orchestrate insect metamorphosis.

Biochemical Properties of EcR/USP

Numerous studies demonstrate that EcR (presumably EcR/USP) binds to radiolabeled ponasterone A (Pon A) with an affinity in the nanomolar range (table 1.1). The binding affinity for a number of ecdysteroids tested are in the order: Pon A > Muristerone A (Mur A) > 20E > ecdysone, with Kd values about one or two orders of magnitude apart between each ecdysteroid. Good correlation has been shown between ecdysteroid-EcR affinity and ED_{50} of ecdysteroid in inducing *Drosophila* imaginal evagination (Yund *et al.*, 1978) or morphogenetic response in Kc cells (Cherbas *et al.*, 1980, see table 1.1).

Using *in vitro* translated *Drosophila* EcR-B1 isoform, Yao *et al* (1993) demonstrated that binding of the EcR to its ligand occurred only in the presence of USP. They also showed that ligand and USP protected EcR from protease digestion, presumably by inducing conformational changes of the EcR. A USP-like protein — BmCF1 was also essential for ligand-EcR binding in *Bombyx mori* (Swevers *et al.*, 1996).

DNA-binding properties of EcR were first demonstrated by non-specific calf-thymus DNA. The latter presumably contains some limited sequences which resemble an ecdysone response element (EcRE). Affinity between *Chironomus tentans* EcR and calf-thymus DNA (Kd = 0.06 nM; Turberg and Spindler, 1992) is about an order of magnitude higher than that of ligand-receptor binding (Kd = 0.65 nM, Turberg *et al.*, 1988). In *Drosophila*, EcR/USP binds to synthetic EcRE with about 1,000 times higher affinity than random DNA sequences, such as calf-thymus DNA (Cherbas and Cherbas, 1996).

EcREs are cis-acting DNA elements at which EcR/USP binds and conveys ecdysteroid response on a target gene. Of several known EcREs, hsp27 EcRE is the most thoroughly studied (Cherbas and Cherbas, 1996). The 23-basepair imperfect palindrome (see table 3.1) was first mapped out by DNase I footprinting analysis and tested by

conferring gene inducibility of ecdysone on a heterologous promoter (Riddihough and Pelham, 1987). Within the dyad, a 15-basepair core sequence (GGGTTCAATGCACTT) is fully functional to convey hormone response in *Drosophila* Kc cells (Cherbas *et al.*, 1991). By testing inducibility of a number of elements, Cherbas *et al.* (1991) proposed a consensus sequence: RRGG/TTCANTGAC/ACYY, an imperfect palindrome containing the DNA half-site AGGTCA for thyroid/ecdysteroid receptor subfamily members.

Using hsp27 EcRE as a radiolabeled probe, DNA-binding properties of EcR/USP were studied in insects by gel mobility shift assay. A number of studies have shown that EcR binds to EcRE only in the presence of USP (Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993; Swevers *et al.*, 1996). Critical nucleotides at positions -6, -5, -2, -1, +2, and +5 as well as one-nucleotide spacer within the palindrome were demonstrated (table 3.1; Ozyhar and Pongs, 1993; Antoniewski *et al.*, 1993). Potentiation of ecdysteroids on EcR/USP-EcRE binding was observed, with Mur A being more potent than 20E (Yao *et al.*, 1993).

Ecdysteroid Hormone Action

Ecdysteroid serves as a polytropic, multifunctional hormone in arthropods including ticks (table 1.2). It is involved in various aspects of arthropod development, behavior and reproduction. Direct effect of an ecdysteroid hormone on gene activation was first reported by Clever and Karlson (1960). They found that ecdysone induces puff formation on polytene chromosomes in *Chironomus* salivary glands within 15-30 min after injection. After observing the puff pattern of salivary gland polytene chromosomes in the presence of 20E *in vitro*, Ashburner *et al.* (1974) proposed a mechanism of ecdysteroid hormone action (figure 1.10). According to this model, 20E induces activity in a small set of early genes and represses activity in a large set of late genes. The protein products of the early genes subsequently repress their own expression, and overcome 20E inhibition of the late genes, the latter being responsible for the biological effects. Therefore, whether a gene

can be activated in the presence of a protein synthesis inhibitor is one criterion to distinguish the early and late gene.

The *ecr* is classified as one of those early genes (Russell and Ashburner, 1996), which are induced by ecdysteroid directly (auto-induction) before the onset of metamorphosis when the ecdysteroid titer is low (Andres *et al.*, 1993; Fujiwara *et al.*, 1995). The *ecr* gene is repressed when ecdysteroid concentration peaks (Karim *et al.*, 1993). In cultured salivary glands of *Drosophila*, 2 nM 20E is sufficient to induce *ecr* mRNA expression (Karim and Thummel, 1992). By adding protein synthesis inhibitor to the culture medium along with 20E, Jindra *et al.* (1996) showed that *Manduca* EcR-B1 mRNA expression was not affected, but its regression was prevented, which further confirmed that EcR is an early gene product.

Isoform-specific antibodies against various N-terminal regions of *Drosophila* EcR have been developed to reveal tissue-specific distribution of EcR during *Drosophila* metamorphosis (Talbot *et al.*, 1993). Various *Drosophila* tissues and organs (imaginal disc, fat body, trachea, salivary gland, ring gland and muscle; Koelle *et al.*, 1991; Talbot *et al.*, 1993) contain EcR, which supports the polytropic role of ecdysteroid hormone. In addition, EcR isoform expression patterns in various target tissues seem to be correlated with the tissue fate during metamorphosis. Tissues destined to die (such as most larval tissues) show strong EcR-B1 antibody staining, whereas tissues surviving and developing into adult structures contain more EcR-A isoform (Talbot *et al.*, 1993).

Ecdysteroid Hormone and EcR in Ticks

Ecdysteroid hormone was first identified in *A. hebraeum* nymphs by Delbecque *et al.* (1978) and the hormone plays several roles in a number of ticks (table 1.2). In both argasid ticks (Zhu *et al.*, 1991) and ixodid ticks (Lomas *et al.*, 1997), the epidermis is the site of ecdysteroid synthesis. In the latter case, a neuropeptide from the synganglion is

known to control ecdysteroid production in the integument. Whether this is also the case for argasid ticks remains to be demonstrated.

A tick EcR with three isoforms (AamEcRA1, A2, and A3) has been recently cloned in *A. americanum* (Guo *et al.*, 1997). The AamEcR DBD and LBD share an average of 86% and 64% identity, respectively, with the corresponding regions of *Drosophila* EcRs. Heterodimerization of AamEcR with AamRXR is also necessary for *in vitro* activation of gene transcription, which suggests a common mechanism of ecdysteroid hormone between insects and ticks. All three EcR isoform mRNAs in the salivary gland increase during feeding and reach their peak level at around the time of engorgement (Guo *et al.*, 1997).

Objectives for the Research of this Thesis

The main objectives of this thesis were (i) to identify an EcR in the salivary gland of *A. hebraeum*, (ii) to determine some of its biochemical properties and (iii) to initiate an understanding at the molecular level of ecdysteroid action on salivary gland degeneration.

This thesis addresses the following questions:

(1) Is EcR present in the salivary gland of ixodid ticks, *A. hebraeum*? If it is, does the EcR bind to ligand in a way resembling EcRs in other arthropods?

(2) Does the tick EcR bind to any known EcRE specifically? Is a USP-like protein required for DNA-binding?

(3) What are the profiles of ecdysteroid hormone and EcR/USP during a feeding cycle? Is there a correlation between hormone, receptor and physiological events in the tissue?

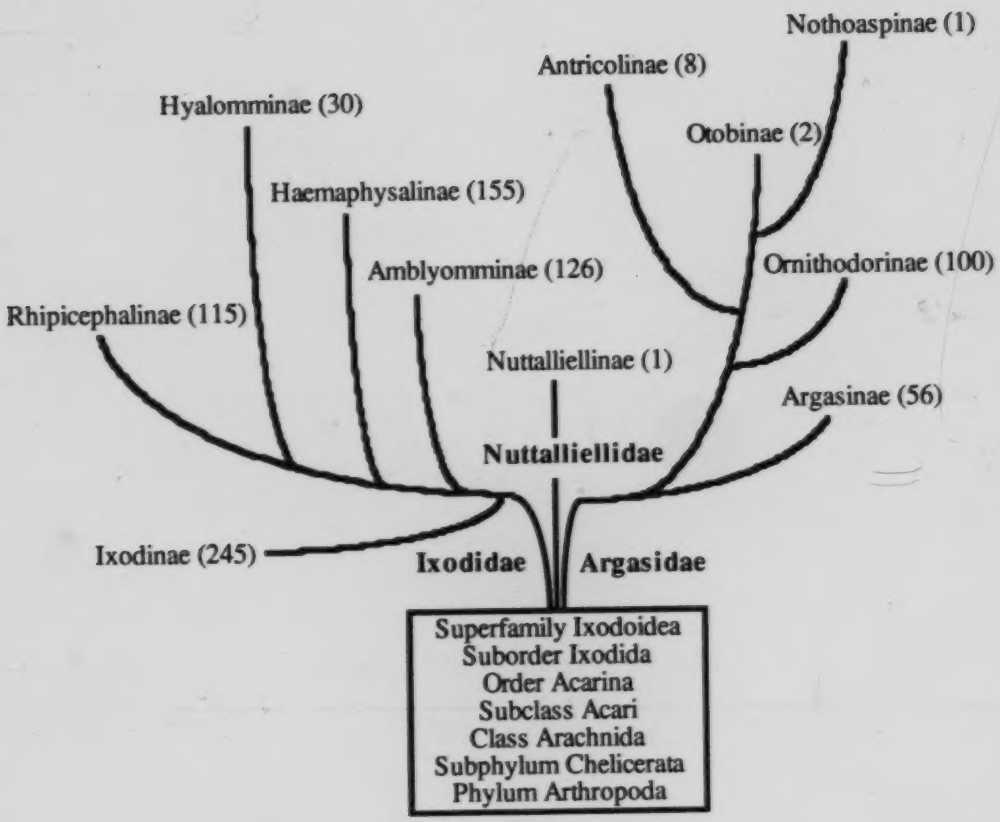


Figure 1.1 Phylogenetic relationship of ixodoidea (modified from Hoogstraal, 1983; Sonenshine, 1991). Number in parenthesis represents number of species in that subfamily.

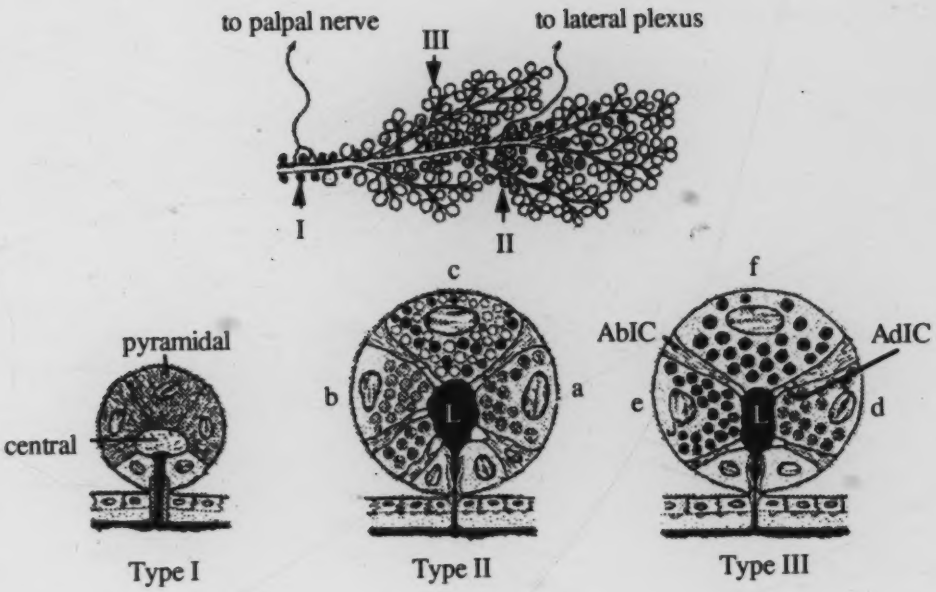
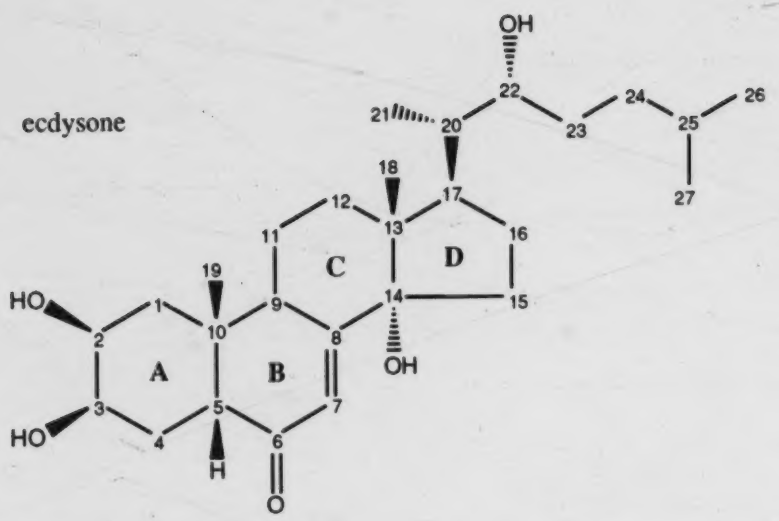
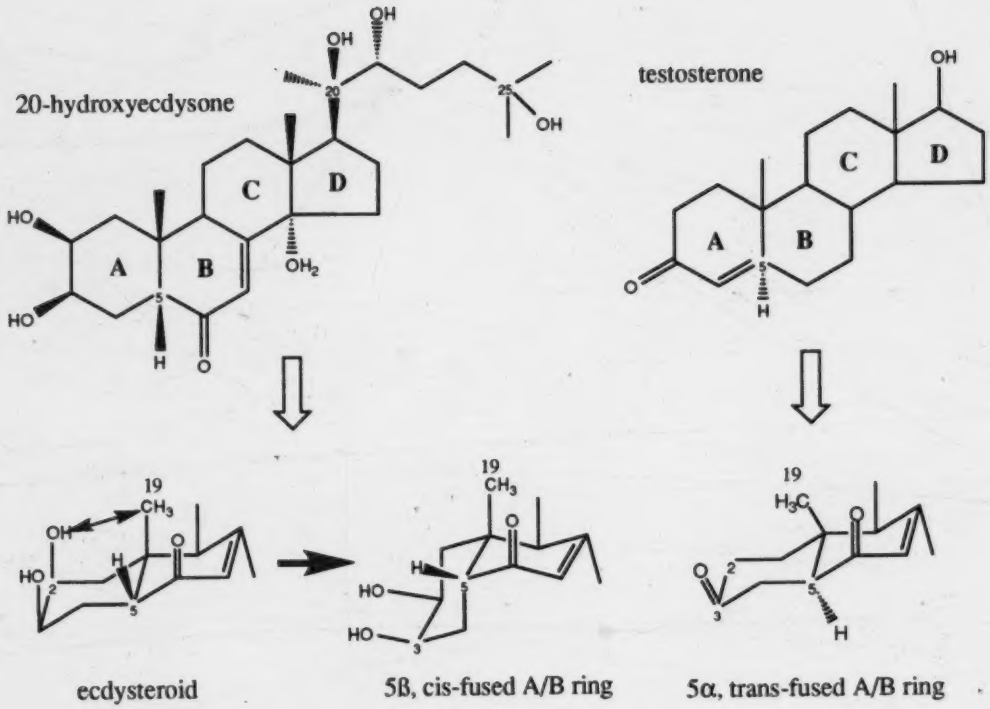


Figure 1.2 Schematic representation of the ixodid tick salivary gland and the 3 types of acini found in the female (modified from Megaw and Beadle, 1979). Lumen (L), abluminal interstitial cell (AbIC), adluminal interstitial cell (AdIC).



Ecdysteroids	C20	C26	C5	C11	C24
ecdysone	—	OH	—	—	—
20-hydroxyecdysone	OH	OH	—	—	—
makisterone A	OH	OH	—	—	CH3
26-mesylinokosterone	OH	OMs	—	—	—
muristerone A	OH	—	OH	OH	—
ponasterone A	OH	—	—	—	—

Figure 1.3 Numbering system and structural features of the ecdysteroids used in the research of this thesis.



	ecdysteroids	vertebrate steroids
5-hydrogen	ß	α
A/B ring junction	*cis*	*trans*
A/B ring configuration	buckled	planar
6-keto-7-ene	yes	no
side chain	long	short
hydrophobicity	low	high

Figure 1.4 Comparison of steroid and ecdysteroid configuration and functional groups.

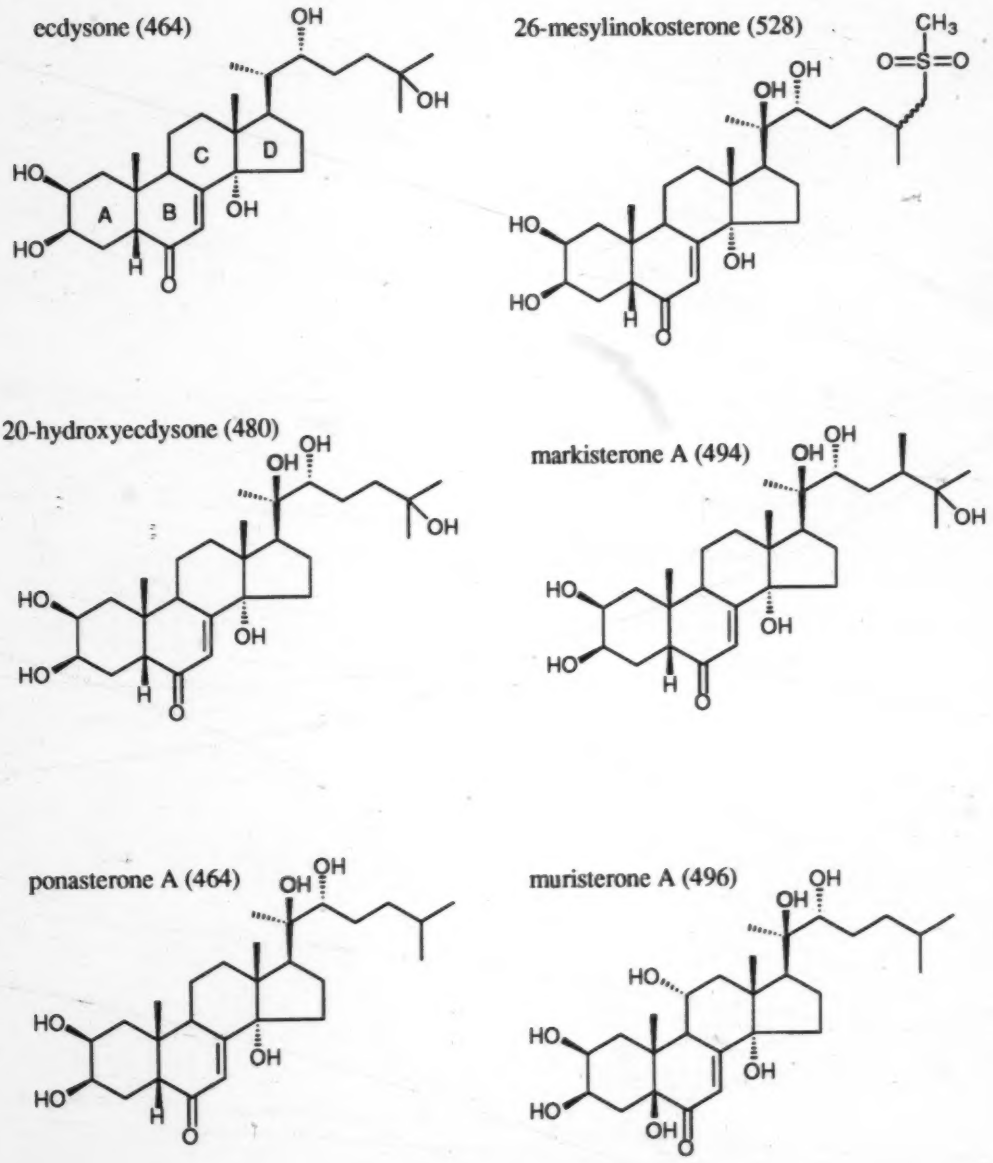


Figure 1.5 Structures of ecdysteroids used in ligand- and DNA-binding studies in this thesis. Numbers in parentheses represent formula weights.

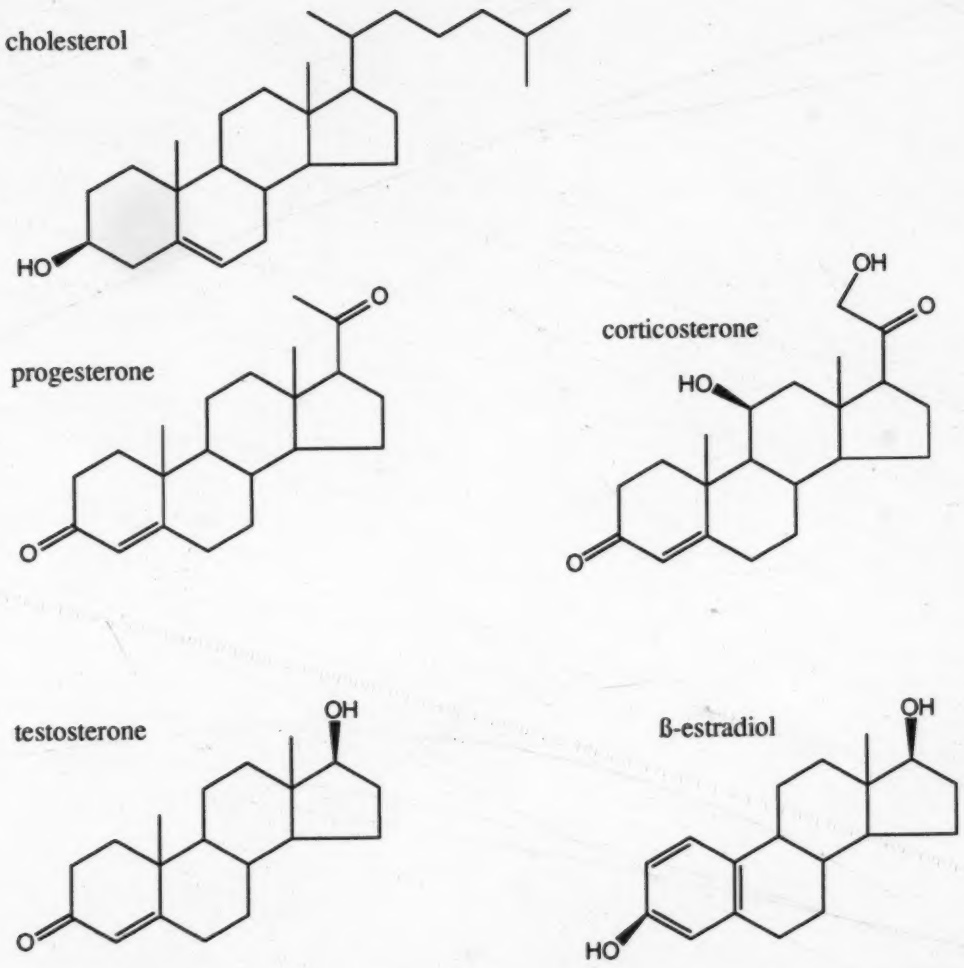


Figure 1.6 Structures of vertebrate steroids used in DNA- and ligand-binding studies in this thesis.

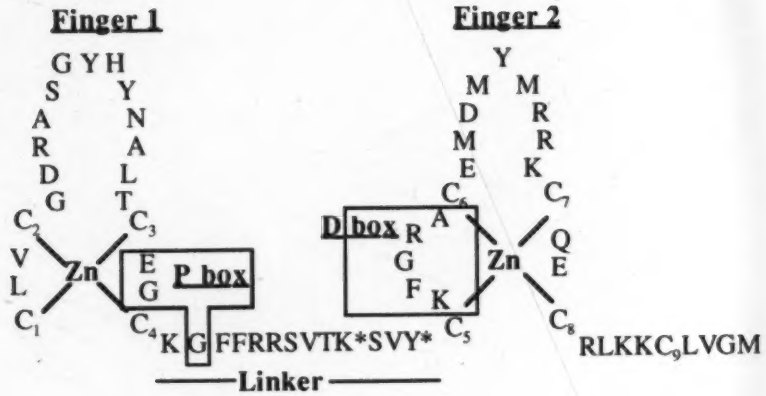


Figure 1.7 Putative C_2C_2 zinc finger structure of the EcR. Deduced amino acids are taken from *D. melanogaster* (Koelle *et al.*, 1991) and *Manduca sexta* EcRs (Fujuwara *et al.*, 1995). The asterisks represent non-conserved amino acid residues.

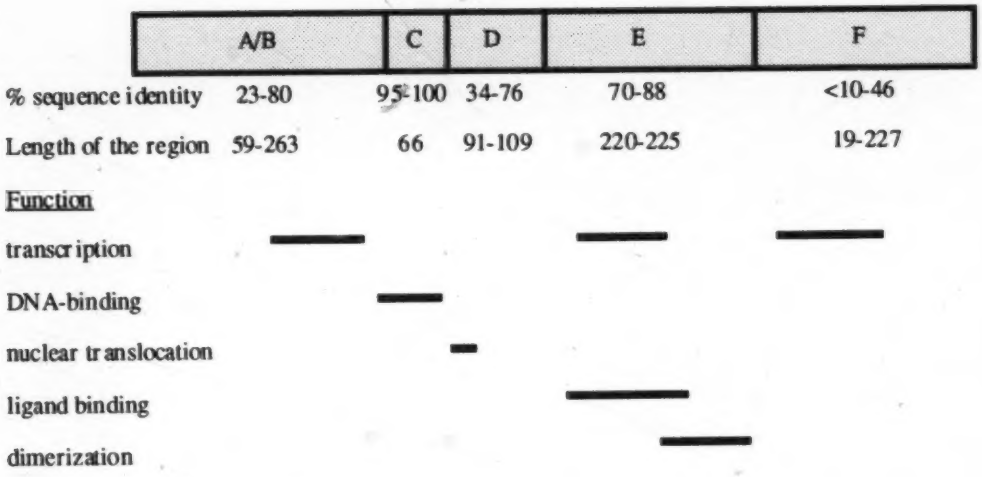


Figure 1.8 Schematic representation of *Drosophila* EcR B1 isoform (modified from Cherbas and Cherbas, 1996). Identity and number of amino acids of each region are taken from Fujiwara *et al.* (1995) based on EcR sequences of *Manduca sexta* (Fujiwara *et al.*, 1995), *D. melanogaster* (Koelle *et al.*, 1991), *Aedes aegypti* (Cho *et al.*, 1995), *Chironomus tentans* (Imhof *et al.*, 1993), *Bombyx mori* (Swevers *et al.*, 1995), and *Choristoneura fumiferana* (Kothapalli *et al.*, 1995).

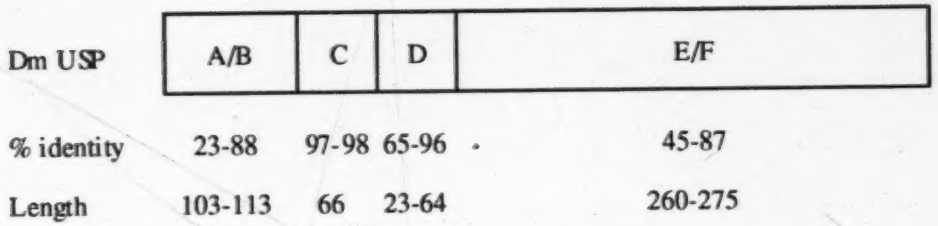


Figure 1.9 Schematic representation of *Drosophila* USP. Identity and number of amino acids in each region are taken from Jindra *et al.* (1997) based on USP sequences of *D. melanogaster* (Henrich *et al.*, 1990), *Bombyx mori* (Tzertzinis *et al.*, 1994) and *M. sexta* (Jindra *et al.*, 1997).

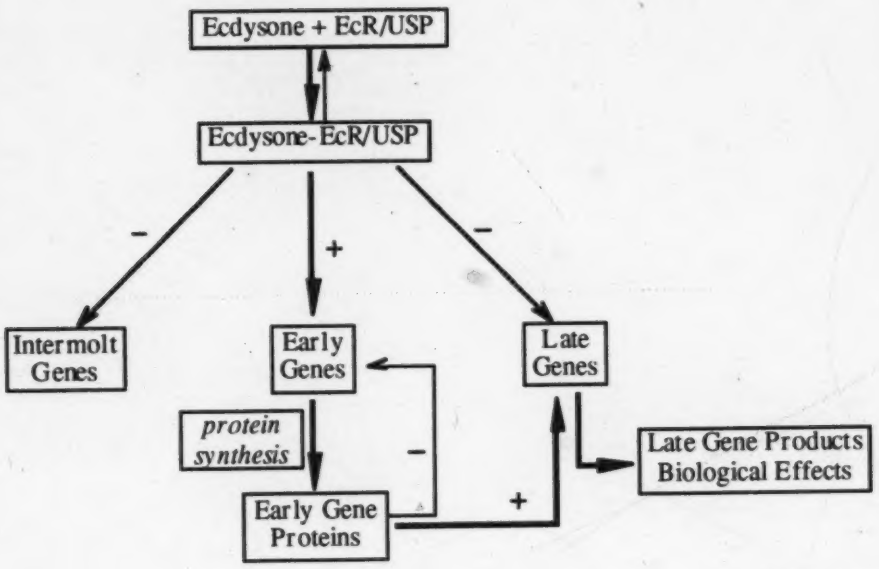


Figure 1.10 Gene regulation of ecdysteroid hormone — Ashburner model (modified from Russel and Ashburner, 1996).

Table 1.1 Summary of Ecdysteroid-EcR Affinity and Biological Activity (Kd, ED_{50} in nM (from Yund and Osterbur, 1985; Bidmon and Sliter, 1990)

Species	Tissue	Pon A	20E	ecdysone	Mur A	ED_{50}†
Drosophila melanogaster	embryo	6	200	50000		
	imaginal disc	2	52	72000		1/42/15000
	pupa	6				
	female	2.9, 0.92*	29			
	Kc cells	0.77-4.2	33-240	1000-36000	15	1.6/20/3300
	BII cells	0.3, 2	200	30000		
Calliphora vicina	3rd instar	1	30	2000		
Chironomus tentans	cell line	0.35	27-50	224		
Orconectes limosus	integument	0.036-0.06	1-3	20-70		
	midgut		1-2	20-30		
Astacus leptodactylus	integument	0.02-0.06				
Artemia salina	Nauplii	9-30				

* Two types of receptor.
† ED_{50} in inducing evagination of imaginal disc or morphogenetic response of Kc cells. The three numbers represent ED_{50} for Pon A, 20E and ecdysone respectively.

Table 1.2 Roles of Ecdysteroid Hormone in Arthropods

Specific effect	Order	Species	References
Cuticulogenesis in embryonic stage	Orthoptera	*Locusta migratoria*	Lagueux et al., 1979
Germ bands growth	Lepidoptera	*M. sexta*	Lanot et al., 1989
& segmentation	Orthoptera	*Blaberus craniifer*	Bullière et al., 1979
Cuticle deposition	Acarina	*Ornithodoros moubata*	Dotson et al, 1991
Embryogenesis	Acarina	*Dermacentor variabilis*	McDaniel and Oliver, 1978
Molting	Lepidoptera	*M. sexta*	Nijhout and Williams, 1974
	Acarina	*Dermacentor albipictus*	Wright, 1969
Apolysis and cuticle formation	Acarina	*Ornithodoros parkeri*	Pound et al., 1984
	Acarina	*Ornithodoros moubata*	Germond et al., 1982
Remolding nervous system	Diptera	*D. melanogaster*	Emery et al, 1994
Inducing wandering behaviour	Lepidoptera	*M. sexta*	Dominick & Truman, 1986
Imaginal disc development	Diptera	*D. melanogaster*	Guillermet & Mandaron, 1980
Oocyte maturation	Orthoptera	*Locusta migratoria*	Lanot et al., 1987; 1988
Vitellogenesis	Diptera	*Aedes detritus* *Aedes caspius*	Guilvard et al., 1984
	Diptera	*D. melanogaster*	Jowett & Postlethwait, 1980
Spermatogenesis	Acarina	*Dermacentor variabilis*	McDaniel & Oliver, 1978
Salivary gland degeneration	Acarina	*A. hebraeum*	Harris & Kaufman, 1985

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Chapter 2

Ligand-Binding Properties of the Ecdysteroid Receptor in the Salivary Gland of the Ixodid Tick, *Amblyomma hebraeum* †

Introduction

In ticks, salivary glands play several important roles in feeding. They secrete (1) the cement-like substance that anchors the tick to the host's skin after attachment (Moorehouse, 1969), (2) anticoagulants to prevent clotting of the host blood (Waxman *et al.*, 1990; Vlasuk, 1993), (3) vasodilators to facilitate the process of imbibition (Chinery, 1981) and, in the case of females, (4) excess water and ions back into the host's circulation (Kaufman, 1989). Finally, during the free living stage, (5) ticks can absorb water vapor from the atmosphere onto a hygroscopic fluid secreted by salivary glands to maintain their osmotic balance (Rudolph and Knülle, 1974; McMullen *et al.*, 1976; Sigal *et al.*, 1991).

During feeding, the salivary glands of females undergo a phenomenal degree of functional development (Kaufman, 1976) accompanied by equally striking ultrastructural changes (Fawcett *et al.*, 1981 a, b). Within 3 to 4 days post-engorgement, the salivary glands of mated females degenerate (Till, 1961), a process triggered by an ecdysteroid hormone (Harris and Kaufman, 1981, 1985; Kaufman, 1991).

Ecdysteroid hormones are also responsible for molting, development and vitellogenesis in arthropods. They act via intracellular receptors which are members of the superfamily of steroid/thyroid hormone receptors and function as "ligand-activated transcription factors", to regulate gene activity (Evans, 1988; Segraves and Hogness, 1990; Koelle *et al.*, 1991). In all inhibition studies conducted to date, the order of ligand affinity for the ecdysteroid receptor (EcR) is ponasterone A > 20-hydroxyecdysone > ecdysone regardless of tissues and species (reviewed by Bidmon and Sliter, 1990; Sobek *et al.*,

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† Paper published in *General and Comparative Endocrinology* **99**, 340-348, 1995.

1993). This correlates well with the order of potency of these hormones *in vivo*. Moreover, crustaceans (which tend to have much lower haemolymph ecdysteroid titers than insects) also exhibit much lower Kd values for their receptors (Londershausen and Spindler, 1981; Londershausen *et al.*, 1982).

Tick salivary glands undergo degeneration after engorgement, and this degeneration can be induced by ecdysteroid hormones both *in vivo* and *in vitro* (Harris and Kaufman, 1981, 1985). The objective of this study was to confirm the existence of an EcR which could play a role in mediating autolysis of the salivary glands and to determine some of its ligand-binding properties using [^{3}H]ponasterone A (Pon A).

Materials and Methods

Ticks

All stages of ticks, *Amblyomma hebraeum* Koch, were reared in the laboratory on rabbits according to the method of Kaufman and Phillips (1973), and held in darkness at 26°C, relative humidity > 95%. Adult males and females were fed together because mating occurs on the host.

Preparation of cytosolic and nuclear fractions

All steps were carried out at 4°C and based on published methods (Fentie *et al.*, 1986; Lehmann and Koolman, 1988). Salivary glands from 15-20 large partially fed ticks (250-500 mg; the stage at which the gland is most developed) were homogenized in TEG buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, pH 7.5 at 20°C) in a glass homogenizer and centrifuged at 4,000 x *g* for 2 min.

The nuclear fraction was prepared from the pellet (see below). The supernatant was further centrifuged at 100,000 x *g* for 1 h (Beckman L2-65B ultracentrifuge, SW55 rotor) to produce a 'high speed cytosol'. The nuclear pellet was re-suspended in 1 ml of cold TEG buffer, pelleted at 8,000 x *g* for 2 min and the supernatant discarded. This procedure

was repeated and 1 ml TEGK buffer (TEG buffer plus 0.6 M KCl, pH 7.5 at 20°C) was added and incubated for 30 min. After centrifugation (13,000 x *g*, 5 min), the supernatant was saved and the procedure was repeated. The supernatants 'nuclear extract', were pooled and dialyzed for 1 h against two changes of 500 volumes of TEG buffer. The desalted nuclear extract was used for the receptor assay.

Stability of radiolabelled ligand

Stability of [^{3}H]Pon A (24,25,26,27-^{3}H(N) Pon A, 195.2 Ci/mmol, NEN Dupont) against potential degradative enzymes in the cytosol in the ligand binding assay was tested by incubating the ligand with 4-fold volume of cytosol at 20°C or 4°C. Samples of the incubation were stopped at various times by adding a 4-fold volume of absolute methanol. After centrifugation at 13,000 x *g* for 5 min, the supernatant was evaporated under vacuum and absolute methanol added. A 10-20 µl aliquot of the resulting solution accompanied with authentic unlabelled Pon A, 20-hydroxyecdysone (20E) and ecdysone was injected on a RP-HPLC column (Radial-Pak µBondapak C18, 8 x 10 mm, solvent was methanol: water, 1: 1, flow was isocratic at 2 ml/min). Radioactivity of each fraction (1 ml) was measured and absorbance at 254 nm was recorded.

Ligand binding assay

Aliquots (0.12 ml) of cytosol or nuclear extract were incubated at 20°C for 5 h with [^{3}H]Pon A at a concentration range of 0.13 to 13 nM in the absence or presence of a 300-fold excess of unlabelled Pon A. The samples were cooled to 4°C and 0.3 ml of dextran-coated charcoal (DCC, 1 mg/ml dextran 500, 1 mg/ml BSA with 10 mg/ml charcoal, Sigma, in TEG buffer) was added. The slurry was vortexed, incubated for 2 min at 4°C, and pelleted at 13,000 x *g* for 5 min. Radioactivity of the resulting supernatant (0.3 ml) was counted on a Beckman LS6500 Scintillation Counter. Receptor affinity and concentration were determined by Scatchard or Woolf analysis (Scatchard, 1949; Kreightley and Cressie, 1980; Kreightley *et al.*, 1983).

Sensitivity of receptor to enzymes, heat and N-ethylmaleimide

Cytosol was incubated with 1 nM [^{3}H]Pon A ± 200-fold excess of unlabelled Pon A in the presence of one of the following treatments: pronase E (0.05 mg/ml, Sigma), DNase I or RNase A (both at 0.1 mg/ml, Sigma; RNase: 59 Kunitz units/mg protein), 5 mM N-ethylmaleimide (Sigma) or heating cytosol at 60°C for 5 min before incubation. DCC was added at the end of 5 h incubation (as above) and radioactivity of the resultant supernatant was measured.

Kinetics of Pon A association and dissociation

Thermo-stability of the ligand-receptor complex was tested by incubating 1 nM [^{3}H]Pon A with cytosol under 4°C or 20°C and stopping with DCC at various times (4 to 36 h at 4°C; 20 to 240 min at 20°C). Association kinetics were tested (methods based on Turberg *et al.*, 1988; Lehmann and Koolman, 1988) by stopping the incubation at various times early in the reaction (2 min to 30 min) in order to estimate the initial reaction rate. Dissociation kinetics were determined by adding 500-fold excess of unlabelled Pon A to the incubation medium after equilibration with [^{3}H]Pon A was achieved, and the incubation was terminated at various times (5 min to 240 min) by adding the DCC suspension.

Competition for [^{3}H] Pon A binding by various steroids

To address the specificity of ecdysteroid and vertebrate steroid binding for the receptor, a binding inhibition experiment was done by incubating cytosol with an aliquot of 1 nM [^{3}H]Pon A in the presence of a series of increasing concentrations of an unlabelled steroid for 5 h at 20°C, followed by DCC adsorption.

Sucrose density gradient centrifugation

Cytosol was incubated with 10 nM [^{3}H]Pon A ± 200-fold excess of unlabelled Pon A at 20°C for 5 h. The incubation was stopped by adding DCC at 4°C and centrifuging. Sixty microliters of the resulting supernatant was mixed with 2 µl of [^{14}C]bovine serum

albumin (BSA, 23.9 μCi/mg, NEN Dupont) and loaded on a 10 - 40% linear sucrose density gradient (4.5 ml) and centrifuged at 240,000 x *g* for 20 h at 4°C (Beckman L2-65B ultracentrifuge, SW55 rotor). The resulting fractions were collected by puncturing the bottom of the centrifuge tube and radioactivity of each fraction (6 drops) was measured by liquid scintillation counting.

Protein concentrations of cytosols were measured according to Lowry *et al.* (1951) using BSA as the standard.

Statistics

Data are reported as mean ± SEM (n). Statistical analysis (Student's t-test) was done using StatView 4.01® Software (Abacus Concepts, Inc.) on a Macintosh computer.

Results

Stability of radiolabelled ligand

Following incubation of [^{3}H]Pon A in cytosol at 20°C (4 h) or at 4°C (22 h), more than 99% of the radioactivity co-migrated with authentic Pon A (results not shown). Thus no detectable enzymatic activity against the ligand is present in the 'high speed cytosol' preparation.

Sensitivity of specific binding to enzymes, heat, and N-ethylmaleimide

Specific binding activity was completely abolished by pronase treatment or heating to 60°C for 5 min; DNase or RNase did not significantly reduce specific binding (figure 2.1). This indicates that Pon A apparently binds to a protein in the tissue and not directly to DNA or RNA. Sensitivity of the protein to N-ethylmaleimide indicates that sulfhydryl groups of the protein are critical for ligand receptor binding.

Kinetics of Pon A binding

Binding equilibrium was achieved about 10 times faster at 20°C than at 4°C (figure 2.2). In addition, maximal binding was maintained for at least another 2 h at 20°C. The association and dissociation kinetics of [^{3}H]Pon A receptor binding at 20°C are shown in figure 2.3. The rate constants, ka and kd, derived from slope of the lines, were 4.6 x 10^6/M, min and 3.1 x 10^{-3}/min, respectively. The half-time for displacing [^{3}H]Pon A from the receptor was about 4 h.

The Kd was determined by three methods: (1) direct calculations of Kd from the association and dissociation rate constants reported above (Kd = kd/ka), (2) from conventional Scatchard analysis (figure 2.4) and (3) from an inhibition experiment (figure 2.5; Rodbard, 1973). The 3 estimates are in very reasonable agreement (table 2.1); the low Kd (low nM range) indicates that the receptor has high affinity for [^{3}H]Pon A.

Saturation of specific binding at 20°C and a Scatchard plot are shown in figure 2.4. The straight line obtained in the Scatchard plot (figure 2.4 inset) indicates a single population of receptor. Average Kd and Bmax values obtained from 8 similar cytosol preparations were 0.72 ± 0.09 nM and 175 ± 12 fmol/mg protein, respectively, at this physiological stage (large, partially fed female ticks).

From 3 nuclear extracts, the Kd and Bmax of the nuclear fraction were 1.1 ± 0.5 nM and 282 ± 35 fmol/mg protein, respectively. These values were not significantly different from those of the cytosol receptor shown just above (Student's t-test).

Binding specificity of ligands

The order of ecdysteroid affinity can be estimated from their Kd values (table 2.1) derived from inhibition experiments (figure 2.5). That is: Pon A > muristerone A > makisterone A > 20E > mesylinokosterone > ecdysone. In contrast, none of the vertebrate steroids tested (estradiol, progesterone, testosterone and corticosterone) displaced a significant amount of Pon A binding even at a 10^5-fold higher concentration (figure 2.6).

Sucrose density gradient centrifugation

Results from a linear 10 - 40% sucrose density gradient using [^{14}C]BSA as the internal standard (figure 2.7) show a 3.2 S binding component in the cytosol fraction. This binding activity could be abolished by adding a 200-fold excess of unlabelled Pon A.

Discussion

There is a moiety in crude extracts of tick salivary glands that binds to [^{3}H]Pon A with high affinity, limited capacity and high specificity. Binding is sensitive to pronase or heating, but not to DNase or RNase, suggesting that [^{3}H]Pon A binds to a protein. The binding protein from the cytosol fraction migrates in the 3.2 S range in a linear 10 - 40% sucrose density gradient. Sedimentation coefficients derived from insects vary from 4 - 4.3 S for cytosolic receptor, 5.9 - 6.3 S for nuclear receptor and 5 S for both fractions in crustaceans (Bidmon and Sliter, 1990). All these properties strongly suggest that the Pon A binding protein is an EcR.

The Kd ($\approx$ 1 nM) for [^{3}H]Pon A binding to the EcR in the salivary gland of *A. hebraeum* is in the range between insects and crustaceans (Bidmon and Sliter, 1990). The estimated Kd for 20E (48 nM) correlates well with the potency of this ecdysteroid in triggering salivary gland degeneration. In organ culture, a significant degree of degeneration can be elicited by 60 nM, and a maximal effect occurs at $\geq$ 400 nM (Harris and Kaufman, 1985; Kaufman, 1991; Lomas and Kaufman, 1992).

In tick salivary gland, there is a significant proportion of receptor in the nucleus during a part of the feeding cycle at which hormonal levels in the haemolymph are quite low (< 20 nM; Harris and Kaufman, 1985; Kaufman, 1991). This is in agreement with receptor localization studies in other arthropods, which show that the EcR resides primarily in the nuclei (Bidmon and Koolman, 1989; Bidmon *et al.*, 1990).

Structure-activity relationships for ecdysteroids in arthropods were reviewed extensively by Horn and Bergamasco (1985). Structural characteristics essential for

ecdysteroid activity are: (1) a *cis*-fused A/B ring configuration, (2) a 6-keto-7-ene grouping in the B ring and (3) a full sterol side chain. Some modifications of the side chain (*e.g.*, 20-OH) enhance activity, whereas others (*e.g.*, 25-OH) diminish it. I categorized the ecdysteroids tested on the tick salivary gland receptor arbitrarily into 3 groups: high affinity (Kd < 10 nM), medium affinity (Kd = 10 - 100 nM) and low affinity (Kd > 100 nM). In table 2.2, I list the ecdysteroids and steroids in descending order of affinity as determined in inhibition experiments (figure 2.5), and indicate their structural characteristics. This table correlates very well with the criteria for ecdysteroid activity presented by Horn and Bergamasco (1985). Unfortunately, the potencies of all these steroids in causing salivary gland degeneration *in vitro* have not yet determined. However, we do know that vertebrate steroids do not cause salivary gland degeneration *in vitro* (Lindsay and Kaufman, 1988) and, as expected, they had no affinity for the EcR (figure 2.6). Close correlation between binding affinities and physiological potencies of ecdysteroids in organ culture may not be as easy to demonstrate because of the potential for metabolic interconversion among ecdysteroids. For example, limited data in Harris and Kaufman (1985) suggest that ecdysone and 20E are equally potent *in vitro*, even though this study showed a more than 10-fold difference in their affinities for the receptor (table 2.1).

In summary, I have demonstrated the apparent presence of an EcR in the salivary gland of the tick, *A. hebraeum*, and have shown that its ligand-binding properties are similar to other known EcRs.

Figure 2.1 Sensitivity of the [^{3}H]Pon A binding component to heat (60°C; 5 min), pronase E, RNase A, DNase I or 5 nM N-ethylmaleimide (n = 4). Cytosol was incubated with 1 nM [^{3}H]Pon A ± 200 fold excess unlabelled Pon A with the simultaneous chemical treatments conducted at 20°C for 5 h (means ± SEM are shown).

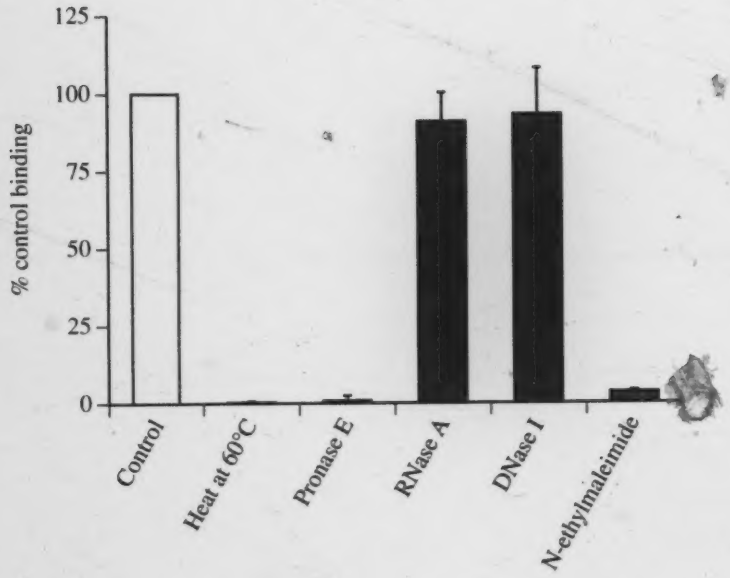
125
100
75
50
25
0
% control binding
Control
Heat at 60°C
Pronase E
RNase A
DNase I
N-ethylmaleimide

Figure 2.2 Association kinetics of specific [^{3}H]Pon A binding at 4°C (closed circles) and 20°C (open circles). Cytosol was incubated with 4 nM [^{3}H]Pon A ± 600-fold excess of unlabelled 20E. At the times indicated, specific binding was determined as the difference between total binding and non-specific binding. There was no significant difference of specific binding at equilibrium between the measurements of the two different temperatures (inset; notice the different time scales).

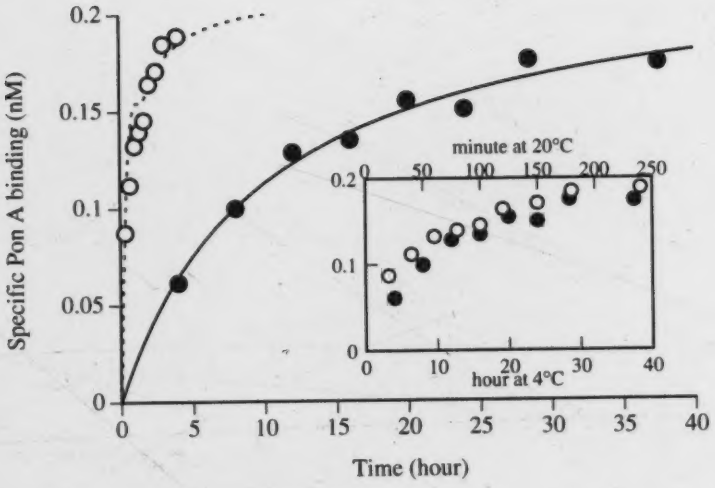
Specific Pon A binding (nM)
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
40
Time (hour)
minute at 20°C
0
50
100
150
200
250
0.2
0.1
0
0
10
20
30
40
hour at 4°C

Figure 2.3 Kinetics of Pon A binding to the receptor. Cytosol was incubated with 1 nM [^{3}H]Pon A ± 500-fold excess of unlabelled Pon A at 20°C. (A) Association kinetics were determined by stopping the incubation at the times indicated. (B) Dissociation kinetics were measured following 5 h incubation with 1 nM [^{3}H]Pon A at 20°C by stopping the incubation at the times indicated after adding 500-fold excess of unlabelled Pon A.

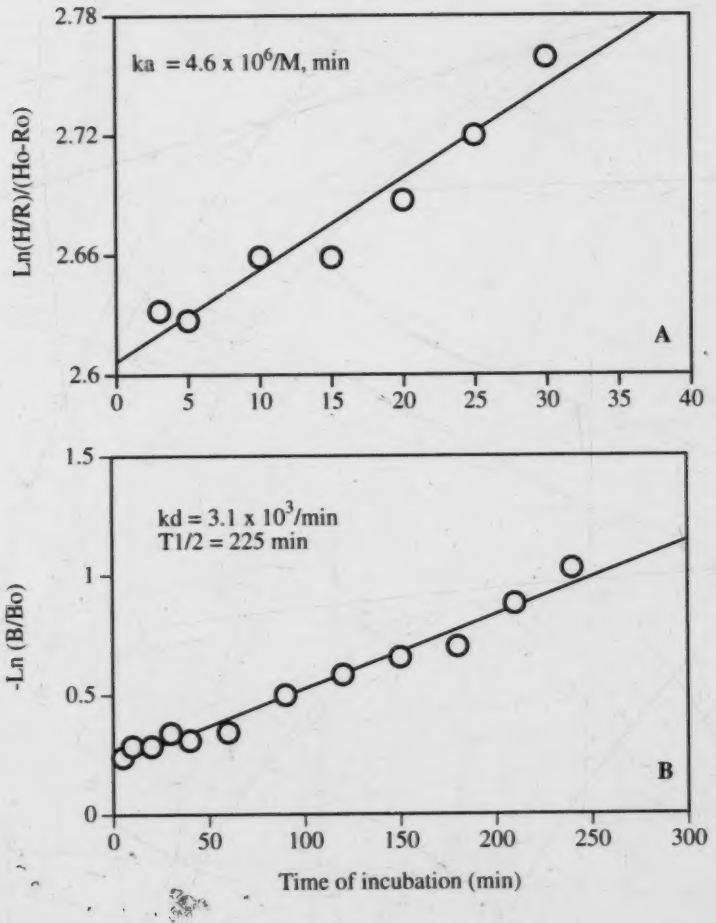
ka = 4.6 x 10^6/M, min
Ln(H/R)/(Ho-Ro)
2.78
2.72
2.66
2.6
A
0
5
10
15
20
25
30
35
40
kd = 3.1 x 10^3/min
T1/2 = 225 min
-Ln (B/Bo)
1.5
1
0.5
0
B
0
50
100
150
200
250
300
Time of incubation (min)

Figure 2.4 Binding of [^{3}H]Pon A to a cytosol preparation. Cytosol (2.3 mg protein/ml) was incubated with increasing concentrations (0.13 to 13 nM) of ligand ± 350-fold excess of unlabelled Pon A at 20°C for 5 h. Specific binding (circles) was calculated by subtracting non-specific binding (triangles) from total binding (squares). The inset shows a Scatchard plot of the same data. Kd and Bmax for this preparation were 1.1 nM and 182 fmol/mg protein respectively.

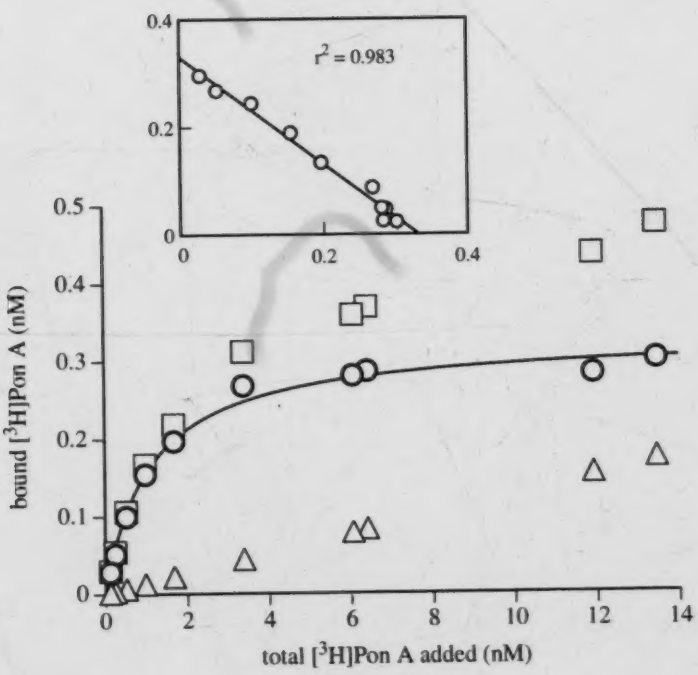
$r^2 = 0.983$
0.4
0.2
0
0
0.2
0.4
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
bound [^{3}H]Pon A (nM)
total [^{3}H]Pon A added (nM)

Figure 2.5 Inhibition of [^{3}H]Pon A binding by unlabelled ecdysteroids. Cytosol was incubated with 1 nM [^{3}H]Pon A ± increasing concentrations of unlabelled ecdysteroid for 5 h at 20°C. The amount of [^{3}H]Pon A binding in the absence of inhibitor was defined as 100%. Relative affinity (estimated from IC_{50}) was in an order: Pon A > muristerone A (Mur A) > makisterone A (Mak A) > 20E > mesylinokosterone (MIS) > ecdysone (E).

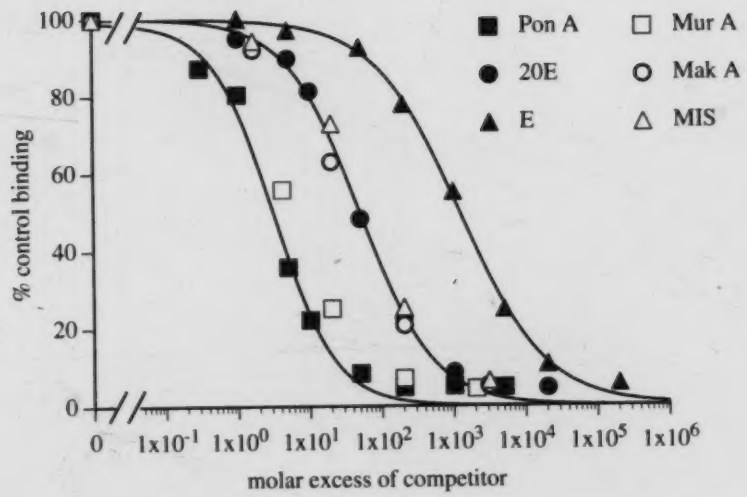
100
80
60
40
20
0
% control binding
0
1x10-1
1x100
1x101
1x102
1x103
1x104
1x105
1x106
molar excess of competitor
Pon A
Mur A
20E
Mak A
E
MIS

Figure 2.6 Inhibition of [^{3}H]Pon A binding by unlabelled vertebrate steroids. Cytosol was incubated with 1 nM [^{3}H]Pon A ± increasing concentrations of unlabelled steroid for 5 h at 20°C. Displacement of unlabeled Pon A binding was copied from figure 2.5. The amount of [^{3}H]Pon A binding in the absence of inhibitor was defined as 100%. Vertebrate steroids tested were estradiol (EsD), testosterone (TTT), progesterone (PgR) and corticosterone (CTT).

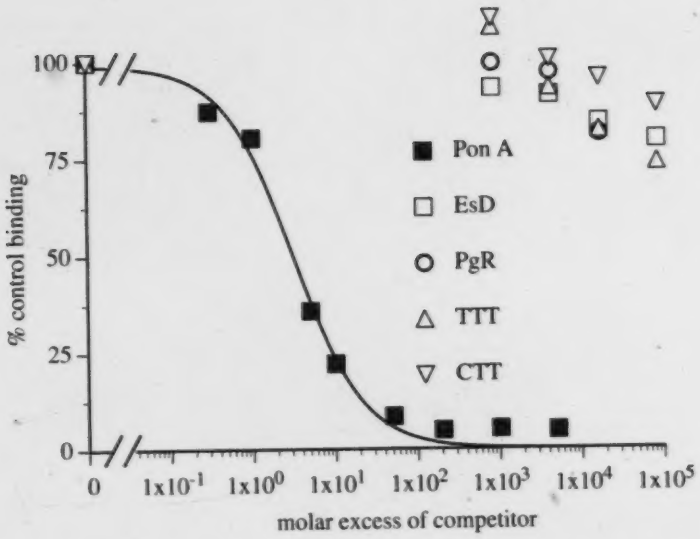
100
75
50
25
0
% control binding
0
1x10-1
1x100
1x101
1x102
1x103
1x104
1x105
molar excess of competitor
Pon A
EsD
PgR
TTT
CTT

Figure 2.7 Sedimentation coefficient for the cytosolic receptor. Cytosol was incubated with 10 nM [^{3}H]Pon A in the absence (closed circles) or presence (open circles) of 200-fold excess of unlabelled Pon A at 20°C for 5 h. The incubation was stopped by adding DCC at 4°C. An aliquot of the supernatant (60 µl) and 2 µl [^{14}C]BSA was layered on 10 - 40% sucrose density gradient and centrifuged at 240,000 x *g* for 20 h at 4°C. The sedimentation coefficient for this receptor (asterisk) was about 3.2 S.

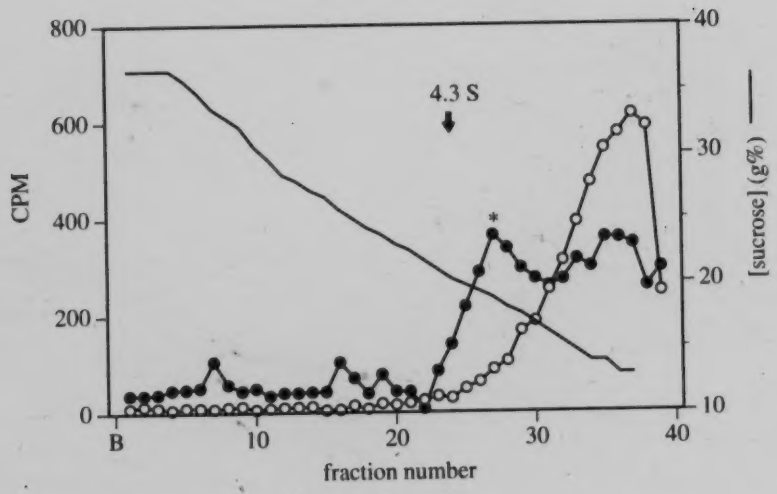
800
600
400
200
0
CPM
4.3 S
*
40
30
20
10
[sucrose] (g%)
B
10
20
30
40
fraction number

Table 2.1 Kd values (nM) of ecdysteroids for EcR using different methods

Ecdysteroid	Kd (Scatchard)	Kd (kinetics)	Kd (inhibition)
ponasterone A	0.72 ± 0.09 (8)	0.74 ± 0.07 (3)	1.7
muristerone A			4.1
makisterone A			28
20-hydroxyecdysone			48
mesylinokosterone			64
ecdysone			658

Table 2.2 Ecdysteroid, steroid structure and binding affinity for EcR

Ecdysteroids Steroids	A/B ring configuration	Modifications of side-chain		Affinity*
		20-OH	25-OH	
Pon A	*cis*-	+	-	high
Mur A	*cis*-	+	-	high
Mak A	*cis*-	+	+	medium
20E	*cis*-	+	+	medium
MIS	*cis*-	+	26-mesyl	medium
ecdysone	*cis*-	-	+	low
ß-estradiol	*trans*-			none
testosterone	*trans*-			none
progesterone	*trans*-			none
corticosterone	*trans*-			none

* Affinities are arbitrarily defined as high (Kd < 10 nM), medium (Kd = 10 - 100 nM), low (Kd > 100 nM).

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Chapter 3

DNA-Binding Properties of the Ecdysteroid Receptor in the Salivary Gland of the Female Ixodid Tick, *Amblyomma hebraeum* †

Introduction

The salivary glands of ixodid ticks (Acari: Ixodidae) play a vital role in both feeding and osmoregulation. Important functions (reviewed by Knülle and Rudolph, 1982; Kaufman, 1989; Sauer *et al*, 1995; Wikel, 1996) include secretion of: (i) an attachment cement which anchors the mouthparts to the skin, (ii) anticoagulants and vasoactive substances which promote blood flow to the feeding site, (iii) immuno-suppressants which attenuate the host's immune response against feeding ticks, and (iv) a hygroscopic saliva responsible for absorbing water vapor from the atmosphere so as to prevent desiccation during the non-feeding stages. A fifth important function, and which has fascinated us for years, is expressed by females: the ability to secrete a large volume of hyposmotic saliva back into the host in order to achieve hemolymph volume- and osmo- regulation. It is because of this large traffic of fluid through the salivary glands that ticks are among the most important transmitters of pathogens to humans and their domestic animals (Nuttall *et al.*, 1994).

For years we have known that the salivary glands of females undergo an enormous degree of development during early feeding stages (Till, 1961; Kaufman, 1976; Fawcett *et al.*, 1981 a, b; Coons and Kaufman, 1988). Following engorgement, the salivary glands degenerate (Till, 1961) due to the action of an ecdysteroid hormone (Harris and Kaufman, 1981, 1985). In a previous study, I demonstrated that [^{3}H]ponasterone A (Pon A) binds to a protein in the tissue extract of salivary glands with high affinity (Kd ≈ 1 nM). The

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† Manuscript submitted for publication.

characteristics of the protein binding to a variety of ecdysteroids indicate an ecdysteroid receptor (EcR).

As a member of the steroid-thyroid hormone receptor superfamily, the EcR binds to its ligand ecdysone and an ecdysone response element (EcRE). Several EcREs have been identified in *Drosophila* (reviewed by Cherbas and Cherbas, 1996), among which hsp27 EcRE has been best studied. The hsp27 EcRE sequence has also been used widely as a specific probe to study DNA binding properties of *Drosophila* EcR-USP, (*ultraspiracle* protein; Ozyhar and Pongs, 1993; Antoniewski *et al.*, 1993).

In this study, I demonstrate that the EcR in the tick salivary gland binds to *Drosophila* hsp27 EcRE in a sequence-specific fashion on a gel mobility shift assay. The binding is influenced by the concentration of KCl and enhanced by ecdysteroids in a dose-dependent manner. Moreover, the hsp27 EcRE binding complex is supershifted by a monoclonal antibody (mAb) against *Drosophila* USP on a gel mobility shift assay.

Materials and Methods

Tick rearing

I used *Amblyomma hebraeum* Koch, from our laboratory colony (26°C, > 95% humidity). Male and female ticks (unfed ticks were in the range of 25 - 30 mg) were fed together on rabbits, because copulation occurs only when adults are feeding on a host. Females were detached forcibly when they reached 200-700 mg, a range at which protein content in the glands reaches a maximum (figure 4.1). The weight at full engorgement is usually 1500-2500 mg.

Preparation of salivary gland tissue extract for the binding assays

Salivary glands from the partially-fed females were dissected in TEG buffer (20 mM Tris, 1 mM EDTA, 10% glycerol, pH 7.6), pooled (3-4 pairs) and stored at -70°C prior to the extraction procedure. The glands were homogenized in various buffers (about

50 µl/gland) in a glass homogenizer. TEGD buffer (TEG buffer with 5 mM dithiothreitol, pH 7.6) was used in the calf-thymus DNA binding assay and western blot analysis. HEGDK buffer (40 mM HEPES, pH 7.6 at 22°C, 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.1 M KCl) was used in the ecdysteroid effect, antibody supershift and oligonucleotide competition experiments. HEGD buffer was used in the experiments testing the effect of KCl on the binding between 20E (1 µM final concentration), tick EcR and hsp27 EcRE, in which KCl (1.5 M) was added in various amounts to make the desired concentration. In all experiments, the homogenate was centrifuged at 70,000 *g* for 45 min and the supernatant used immediately or stored at -70°C. Henceforth, this preparation is referred to as 'tissue extract'. Protein concentration (usually 2-3 µg/µl) of the tissue extracts was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Competitive calf-thymus DNA binding assay

In order to see if the specific Pon A binding protein also binds to hsp27 EcRE, a competitive binding experiment using calf-thymus DNA (CT-DNA) cellulose was performed. Tissue extract was incubated with [^{3}H]Pon A (170 Ci/mmol, NEN Dupont) for 2 h at 22°C in TEGD buffer and treated with dextran-coated charcoal (DCC) for 5 min at 4°C to remove free [^{3}H]Pon A. The DCC-treated supernatant (200 µl) was mixed (orbital shaker; 20 min; 4°C) with CT-DNA cellulose (~15 µg CT-DNA; Sigma) in the absence or presence of CT-DNA (Sigma), synthetic single or double-stranded hsp27 EcRE (see below for preparation). Binding of specific Pon A binding protein to CT-DNA cellulose reached equilibrium within 10 min of incubation, and was maintained for at least 30 min at 4°C (results not shown). Parallel experiments using an equal weight of cellulose (Sigma) were performed to estimate the proportion of [^{3}H]Pon A-EcR bound to CT-DNA; typical values of ligand-receptor bound to cellulose were approximately 20-50% of the amount bound to CT-DNA cellulose.

The slurry was centrifuged at 8,000 *g* for 6 seconds and washed twice with ~1 ml TEGD buffer. Radioactivity of the CT-DNA cellulose pellet was counted on a liquid scintillation counter.

Recognition of tick EcR and USP-like proteins by Drosophila mAbs

Tissue extract in TEGD buffer was mixed with 2X sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 0.001% bromphenol blue, and 10% ß-mercaptoethanol added just before use) and boiled for 5 min. The mixture was resolved by 7.5% SDS-polyacrylamide gel electrophoresis at 90 volts using a mini-gel apparatus (BioRad). The gel was transferred to a PVDF membrane (BioRad) at 100 volts for 40 min and blocked with 5% skimmed milk powder/2% BSA in TTBS buffer (30 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.5) for 2 h at 4°C. The membrane was immunoblotted with 1:50 *Drosophila* mAb against USP (AB11; Khoury Christianson *et al.*, 1992) obtained from Dr. F. C. Kafatos, European Molecular Biology Laboratory, Heidelberg, Germany or 1:10 *Drosophila* mAbs against EcR common regions (AG9.2, JG6.2, EEC11.1, DDA2.7, IID9.6, AAA5.4, AC12.4 and AG10.2; Talbot *et al.*, 1993) obtained from Dr. P. Hurban, Stanford University School of Medicine. The membrane was subsequently incubated with biotinylated goat anti-mouse antibody (1:2000, Sigma) and then with streptavidin-conjugated alkaline phosphatase (BioRad). The protein bands were visualized by incubating the membrane with BCIP (5-bromo-4-chloro-3-indoylphosphate p-toluidine salt) and NBT (p-nitro blue tetrazolium chloride, BioRad).

Preparation of single- and double-stranded oligonucleotides and the radiolabeled probe

Synthetic hsp27 EcRE is an imperfect palindromic sequence composed of two half-sites separated by a one-nucleotide spacer (table 3.1). Hsp27 EcRE and a number of mutated sequences (Ozyhar *et al.*, 1991; Ozyhar and Pongs, 1993) were made on an Applied Biosystems 391 DNA synthesizer PCR-MATE according to the manufacturer's

instructions. Each double-stranded oligonucleotide was named according to the nature or position of the mutated nucleotide (table 3.1).

Equimolar concentrations of complementary single-stranded oligonucleotides were annealed in TEK buffer (20 mM Tris, pH 7.6, 1 mM EDTA, 0.1 M KCl) by heating at 75°C for 10 min, and cooled to room temperature over a 2 h period and then stored at 4°C. Concentrations of the single- and double-stranded oligonucleotides were calculated based on the measurements on a spectrophotometer at 260 nm.

Double stranded hsp27 EcRE with a 5' overhang [hsp27 (5'o.h.), table 3.1] was end-labeled with [γ-^{32}P]ATP (4,500 Ci/mmol, ICN) using T4 polynucleotide kinase at 37°C for 1 h. The [^{32}P]hsp27 EcRE was isolated and free [γ-^{32}P]ATP removed by salt and ethanol precipitation. The radiolabeled probe was re-dissolved in Tris-EDTA buffer, pH 7.6 and stored at 4°C.

Gel mobility shift assay

Tissue extract was incubated with [^{32}P]hsp27 EcRE in the presence of poly[dI-dC]•poly[dI-dC] (Sigma) for 1 h at 22°C. In experiments testing the competitive binding of oligonucleotides, or testing the effect of KCl, various ecdysteroids and vertebrate steroids, these substances were added to tissue extract/[^{32}P]hsp27 EcRE mixture in the amounts necessary to achieve the concentrations indicated in the figures. The final maximal concentration of ethanol in the incubation mixture was 2% for vertebrate steroids and 0.7% for the ecdysteroids. The antibody supershift assay was performed by adding 1 µl *Drosophila* antibody against USP and/or against EcR common regions to the tissue extract/[^{32}P]hsp27 EcRE incubation.

For all the above, the binding mixture was resolved on a 4% pre-run (100 volts for 90 min at 22°C) native polyacrylamide (1:39 of bis:acrylamide) gel in 0.5X TBE buffer at 140 volts for 2 h at 22°C. Gels were dried at 60°C for 2 h and exposed to X-ray film (X-

OMAT™-AR, Kodak Scientific Imaging Film, New York). Relative band intensity was estimated by a PhosphorImager™ analyzer (Molecular Dynamics) and ImageQuant™ software on a IBM computer. Relative band intensity was expressed as arbitrary units generated by the program.

All quantitative data are reported as mean ± SEM (n).

Results

Displacement of Pon A binding to CT-DNA cellulose by double-stranded hsp27 EcRE

Figure 3.1 shows that the specific Pon A binding to CT-DNA cellulose can be displaced by CT-DNA and by synthetic double-stranded hsp27 EcRE in a dose-dependent manner. In contrast, neither of the single complementary strands of hsp27 EcRE displaced Pon A binding from CT-DNA cellulose. Relative affinity of CT-DNA and hsp27 EcRE cannot be estimated from the curves because the CT-DNA fragments were not of uniform size.

Binding of salivary gland tissue extract to hsp27 EcRE

Figure 3.2A shows the binding of the tissue extract to [^{32}P]hsp27 EcRE on a gel mobility shift assay. A retarded band could be seen only when the tissue extract (4-128 μg protein) was added. The band intensified as the amount of protein increased. Virtually all the free probe was shifted at the higher protein levels (64 - 128 μg). Figure 3.2B shows the increase in intensity of the retarded band as measured by the PhosphorImager™ analyzer.

KCl is required for, and ecdysteroids enhance, the binding of tick EcR to hsp27 EcRE

It has been shown that KCl plays an essential role in the binding of vitamin D_3 receptor-retinoid X receptor and its response element, VDRE (Kimmel-Jehan *et al.*, 1997). EcR/USP binding to hsp27 EcRE was also greater in KCl-treated tissue extract of *Drosophila* S3 cells (Luo *et al.*, 1991). Thus, in order to see if KCl affects the formation of

the hsp27 EcRE complex, I tested a series of increasing concentrations of KCl on a gel mobility shift assay. The tissue extract did not bind to [^{32}P]hsp27 EcRE in the absence of KCl, irrespective of the presence of 1 μM 20E (figure 3.3, lanes 1 and 2). Binding was apparent in the presence of 25 mM KCl, and peaked at 75 mM KCl (lanes 3 - 8). Binding decreased thereafter. In all cases, there was a noticeable enhancement of binding in the presence of 20E. When KCl exceeded 150 mM, there was virtually no binding in the absence of 20E; some binding was rescued at high KCl, however, when 20E was present (lanes 16, 18, 20). This experiment was repeated twice more with similar results.

I then tested if this effect is ecdysteroid-specific. Other ecdysteroids with a significant affinity for the tick EcR (Mao *et al.*, 1995) and able to trigger salivary gland degeneration *in vitro* (Lindsay and Kaufman, 1988; Kaufman, 1991) are ecdysone (E), makisterone A (Mak A), muristerone A (Mur A), and Pon A. There was a significant degree of binding in the absence of the exogenous ecdysteroid (figure 3.4A, figure 3.4B lanes labeled "0"). All five ecdysteroids intensified the shifted band in a dose-dependent manner. None of the vertebrate steroids (ß-estradiol, cholesterol, corticosterone, progesterone, testosterone) potentiated the binding even at much higher concentrations (0.02 - 200 μM, figure 3.4B). Thus, the potentiation of binding is specific for ecdysteroids. Figure 3.4C shows that the potencies of the ecdysteroids are in the order: Pon A > Mur A > Mak A > 20E > E.

The binding complex contains a USP-like protein

USP is an essential component for both ligand- and DNA-binding of EcR in insects (Yao *et al.*, 1992; 1993; Thomas *et al.*, 1993; Swevers *et al.*, 1996). I screened a number of mAbs from *Drosophila* in order to identify those which might recognize a tick EcR and USP-like protein. Only AB11 (raised against the USP DNA-binding domain) and AG10.2 (raised against the EcR F region; figure 3.8) recognized discrete bands from tissue extract by western blot analysis (figure 3.5). Other antibodies against various EcR common

regions (figure 3.8) did not recognize any band under the same experimental conditions (results not shown). The molecular masses of the three bands detected by AG10.2 (figure 3.5) were 90.5 ± 0.8, 87.3 ± 0.8 and 84 ± 0.8 kDa (n = 18). Two major bands recognized by AB11 were 50.3 ± 0.5 and 47.1 ± 0.5 kDa (n = 17). There was no cross reaction between the two antibodies (results not shown).

In order to see if a USP-like protein participates in the DNA binding of tick EcR, gel mobility shift assay was performed using the antibodies mentioned above. Only AB11 supershifted the [^{32}P]hsp27 EcRE-tick EcR complex band (figure 3.6, lane 3), indicating that the binding complex contains USP-like proteins. None of the *Drosophila* antibodies directed against the EcR common regions, including AG10.2 (figure 3.6, lane 2), supershifted the complex band. Likewise, there was no additional effect (*i.e.*, a super-supershifted band) when including both AG10.2 and AB11 in the binding mixture (figure 3.6, lane 4).

The tick EcR/USP binds specifically to hsp27 EcRE sequence

In order to see if the tick EcR/USP binds to the hsp27 EcRE sequence specifically, I performed a competitive gel mobility shift assay using unlabeled hsp27 EcRE and its mutated oligonucleotides (table 3.1). All the synthetic oligonucleotides could displace [^{32}P]hsp27 EcRE from the shifted complex band in a dose-dependent manner and with various potencies (figure 3.7A).

Based on the relative band intensity data measured on the PhosphorImager™ analyzer, I plot the degree of displacement as a function of amount of the competitor added (figure 3.7B). Apparent affinities (IC_{50}) of these oligonucleotides for the tick EcR/USP were calculated on the basis of the best-fit curves. The relative affinities listed in table 3.2 are in the order: hsp27 (5'o.h.) ≈ hsp27 EcRE > hsp27 (17) > hsp27 (-5) ≈ hsp27 (+2) ≈ hsp27 (IV) ≈ hsp27 (S2).

Discussion

This study confirms our earlier finding (Mao *et al.*, 1995) that the salivary gland of female *A. hebraeum* contains an EcR, and demonstrates that the DNA binding properties of this EcR are similar to those of a number of insect EcRs. The fact that it heterodimerized with a USP-like protein and bound specifically to a *Drosophila* EcRE suggests that the mechanism of tick EcR/USP-DNA binding is similar to that of insects. In addition, the tick EcR/USP DNA binding domains and the tick EcRE seem to be highly conserved.

The intranuclear concentration of K^+ in the oocyte of the salamander *Desmognathus* has been estimated at about 144 mM (Paine *et al.*, 1981). In the presence of 1,25-hydroxyvitamin D_3 maximal binding between the vitamin D_3 receptor (VDR) and VDRE occurs at a similar KCl concentration (110-130 mM, Kimmel-Jehan *et al.*, 1997). In the absence of its cognate ligand, maximal binding occurs at about 50 mM KCl. However, in a medium of higher salt concentration (150-170 mM), the presence of ligand is required for binding (Kimmel-Jehan *et al.*, 1997). Treatment of *Drosophila* S3 cell nuclear extract with 0.5 M KCl enhances EcR/USP binding to hsp27 EcRE (Luo *et al.*, 1991). Here I show a similar requirement for KCl in the binding among tick EcR-USP-hsp27 EcRE (figure 3.3). At higher KCl concentrations (150 - 250 mM), tick EcR/USP could not bind in the absence of ligand, although 20E was able to rescue some binding (figure 3.3). I do not know the significance of this slight rescuing effect by 20E.

In all systems studied so far, the ecdysteroid hormone is essential to activate or repress its cognate gene, although the presence of hormone is not necessary for EcR/USP-EcRE binding (reviewed by Cherbas and Cherbas, 1996). Nevertheless, in several insect studies (Luo *et al.*, 1991; Yao *et al.*, 1993; Swevers *et al.*, 1996), exogenous ecdysteroid did enhance the binding. The tick EcR/USP-like proteins showed significant binding to hsp27 EcRE in the absence of exogenous ecdysteroids (figure 3.3). I do not know whether this is an intrinsic feature of the heterodimer or whether it is caused by endogenous ecdysteroids in the tissue extract. The salivary glands of this feeding stage contain a

significant amount of endogenous ecdysteroid (18.8 ± 1.45 ng 20E equivalents/g tissue, n = 24, or ≈ 40 nM, measured by RIA; figure 4.2B). I have not been successful in removing this endogenous ecdysteroid in order to test whether the basal level of binding can be reversed.

The following considerations highlight the physiological relevance of the findings reported here. (1) There is a good correlation between (***a***) the relative affinities of these ecdysteroids for the tick EcR as tested earlier by competitive binding experiments (Mao *et al.*, 1995) and (***b***) the effect of ecdysteroids on enhancing the binding between the tick EcR/USP and *Drosophila* hsp27 EcRE. Table 3.3 illustrates that the ratio between ***a*** and ***b*** (from 0.8 - 3.9) is much smaller than the range of relative concentrations (1.7 - 658 nM for displacing Pon A binding and 1 - 170 nM for enhancing EcR-hsp27 EcRE binding). (2) All the ecdysteroids which promote binding also cause salivary gland degeneration *in vitro* (Lindsay and Kaufman, 1988; Kaufman, 1991). Finally, (3) vertebrate steroids which did not displace Pon A binding in a competitive binding assay (Mao *et al.*, 1995), and which failed to induce salivary gland degeneration *in vitro* (Lindsay and Kaufman, 1988), also had no effect on enhancing tick EcR/USP-DNA binding (figure 3.4B).

It is well known that the *Drosophila* EcR binds to its EcRE only when EcR heterodimerizes with USP (Yao *et al.*, 1992; Thomas, 1993; Swevers *et al.*, 1996). Results from our AB11 supershift assay suggest the same scenario for tick EcR-DNA binding. Both EcR and USP-like proteins are present in the tissue extract of salivary glands, and their molecular masses are similar to those found in other insects (table 3.4). AB11 recognizes part of the DNA binding domain of *Drosophila* USP (Khoury Christianson *et al.*, 1992), which is a highly conserved region among members of steroid-thyroid hormone receptor superfamily. Apparently, the DNA-binding domain of the tick USP-like proteins is conserved as well because AB11 recognizes both the native (in gel mobility supershift assay) and denatured proteins (in western blot). AG10.2 is the only EcR antibody mentioned above that recognizes protein bands in the salivary gland (figure 3.5) and a

number of female tick tissues: synganglion, trachea, integument, ovary, fat body and muscle in western blot (results not shown). However, AG10.2 failed to supershift the complex band in the gel mobility shift assay (figure 3.6). One possible explanation for this is that AG10.2 was raised against *Drosophila* EcR F region (Talbot *et al.*, 1993), the least conserved among the five functional domains (figure 3.8). Thus, it might only recognize the denatured tick EcR in western blots but not the native form in the gel mobility shift assay.

A 23-bp sequence from the hsp27 gene promoter region was identified as an EcRE by DNase I footprinting analysis and confirmed by ecdysone inducibility on a heterologous gene (Riddihough and Pelham, 1987). The 15 basepair core of the element (table 3.1) is a fully functional element as tested in *Drosophila* Kc cells (Cherbas *et al.*, 1991). Based on the latter result, Cherbas *et al.* (1991) proposed a consensus sequence RRGG/TTCANTGAC/ACYY, in which -5(G) and +2(G) are also conserved among vertebrate steroid hormone response elements (GRE, ERE, TRE, summarized in Luo *et al.*, 1991). By testing the binding of site-mutated EcREs within the palindrome for tick EcR/USP, Ozyhar and Pongs (1993) were able to identify some nucleotides which strongly influence the protein-DNA binding. The important bases were at positions -6, -5, -2, -1, +2, and +5 (table 3.1). The identity of the spacer dividing the palindrome was not critical, but deleting it or adding extra bases markedly reduced binding. Similarly, Antoniewski *et al.*, (1993) found that methylating the guanine at position -6, -5 or +2 also markedly reduced binding. In this study, hsp27 EcRE (17), containing the imperfect palindrome flanked on each side by one nucleotide, has 2 - 3 times higher affinity than any single-base mutated EcRE (table 3.2). It suggests that the tick EcRE core region is similar to *Drosophila* hsp27 EcRE. Nucleotides beyond the 17-bp core region may also play a role in stabilizing the complex formation, because the longer version, 27-bp hsp27 EcRE, had 13 times higher affinity than 17-bp hsp27 (17). Furthermore, I found that the hsp27 EcRE mutants with the nucleotide altered at position -5 or +2, or inserting an extra nucleotide in

the spacer region, all resulted in much lower affinity for tick EcR/USP (figure 3.8, table 3.2). These results agree with the studies in insects mentioned above, indicating considerable specificity of the binding between tick EcR/USP and the EcRE. It also suggests that the tick EcRE might be conserved in those positions. Taken together, I predict a sequence similarity of EcREs between *A. hebraeum* and *D. melanogaster*.

In conclusion, this study demonstrates that the tick EcR/USP binds to *Drosophila* hsp27 EcRE in a sequence-specific fashion. The binding is enhanced by ecdysteroids with various potencies. Furthermore, the DNA-binding complex contains a USP-like protein. Combining these results with the ligand binding properties of tick EcR in the earlier study (Mao *et al.*, 1995), I suggest that tick EcR/USP-like protein resembles the *Drosophila* counterparts and other arthropods in terms of ligand- and DNA-binding properties.

Figure 3.1 Competitive binding of synthetic oligonucleotides and calf-thymus DNA for Pon A binding proteins (n = 2 - 4). The tissue extract was incubated with 5 nM [^{3}H]Pon A for 2 h at 22°C. After DCC-treatment to remove free ligand, the supernatant was incubated with CT-DNA cellulose (~15 µg DNA) in the presence of either synthetic double-stranded (d.s.) hsp27 EcRE (squares), single-stranded (s.s.) hsp27 EcRE (triangles) or CT-DNA (circles) for 20 min at 4°C. CT-DNA bound in the absence of competitor is defined as 100%. Two curves were generated by Deltagraph™ software: one being the best-fit for CT-DNA and the other for d.s. hsp27 EcRE.

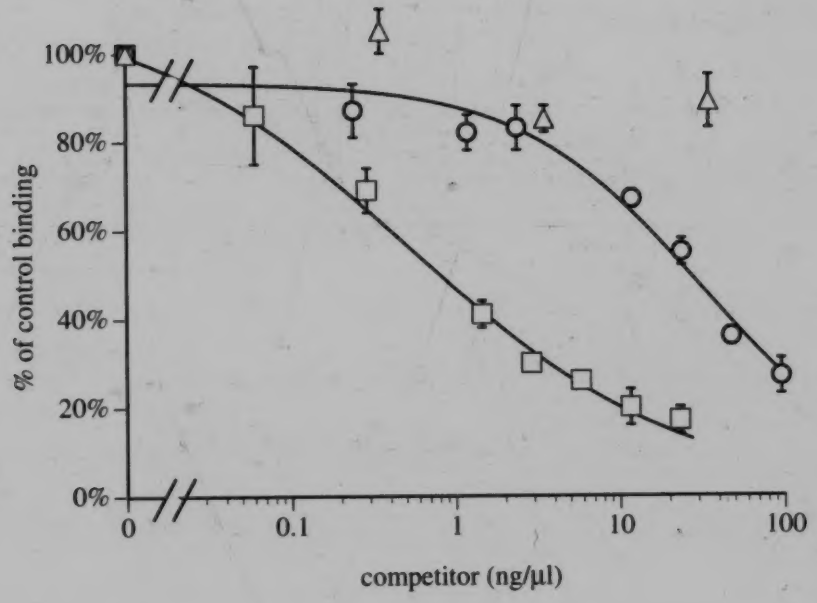
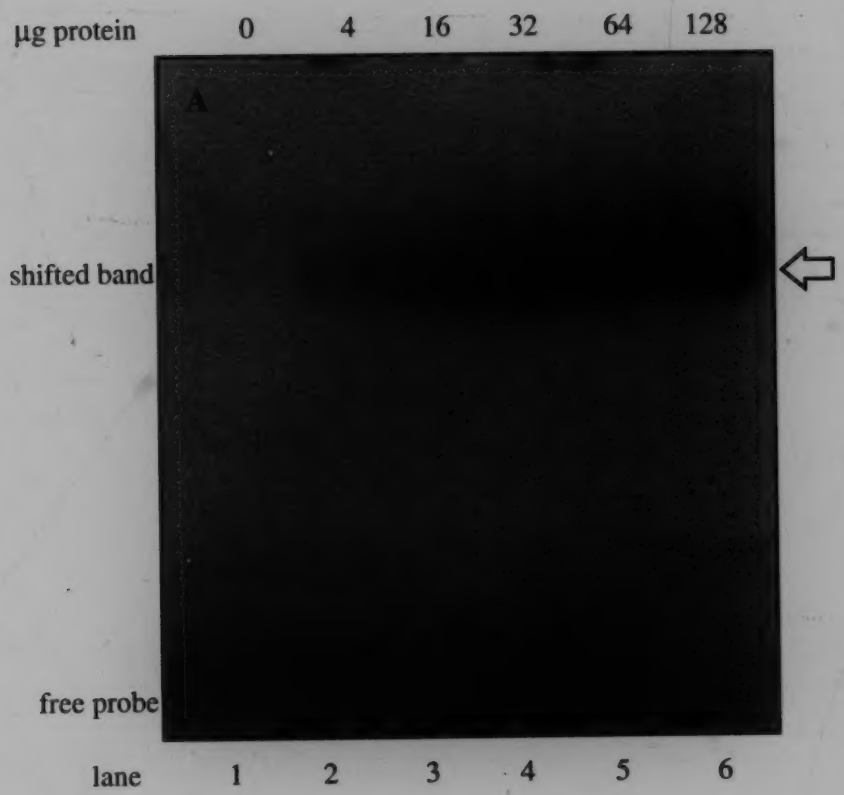

100%
80%
60%
40%
20%
0%
% of control binding
0
0.1
1
10
100
competitor (ng/µl)

Figure 3.2 (A) Gel mobility shift assay of hsp27 EcRE and tissue extract protein binding. Increasing amounts of tissue extract protein were incubated with ~ 25 fmol [^{32}P]hsp27 EcRE along with poly[dI-dC]•poly[dI-dC] for 30 min at 22°C. The mixture was resolved on a 4% polyacrylamide gel for 2 h at 22°C. The arrow indicates the shifted band.

(B) Relative intensity of the retarded band from the PhosphorImager™ analyzer (n = 2). The saturation curve was drawn using Deltagraph™ software.


µg protein
0
4
16
32
64
128
A
shifted band
free probe
lane
1
2
3
4
5
6

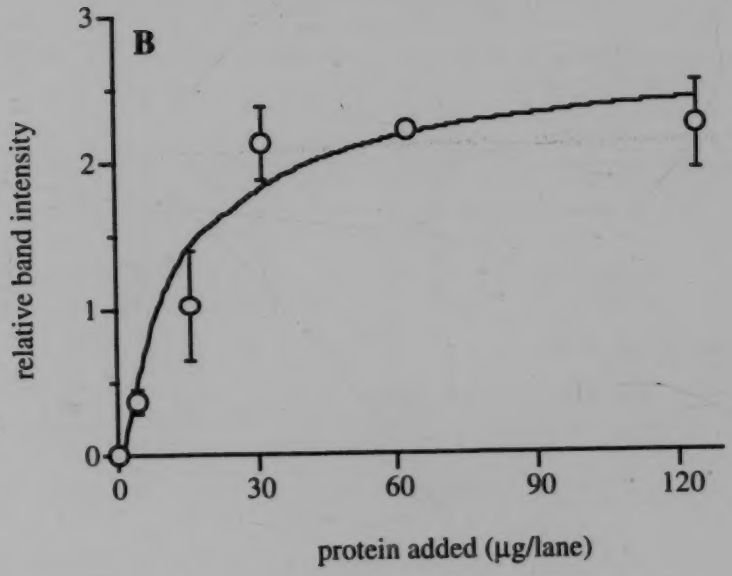

B
relative band intensity
3
2
1
0
0
30
60
90
120
protein added (µg/lane)

Figure 3.3 Effect of KCl and 20E on tick EcR and [^{32}P]hsp27 EcRE complex formation (only the shifted bands are shown). KCl (various amounts of 1.5 M) and 20E (final concentration = 1 μM) were incubated with [^{32}P]hsp27 EcRE and the tissue extract for 1 h at 22°C. The mixture was resolved on a 4% polyacrylamide gel for 2 h at 22°C.

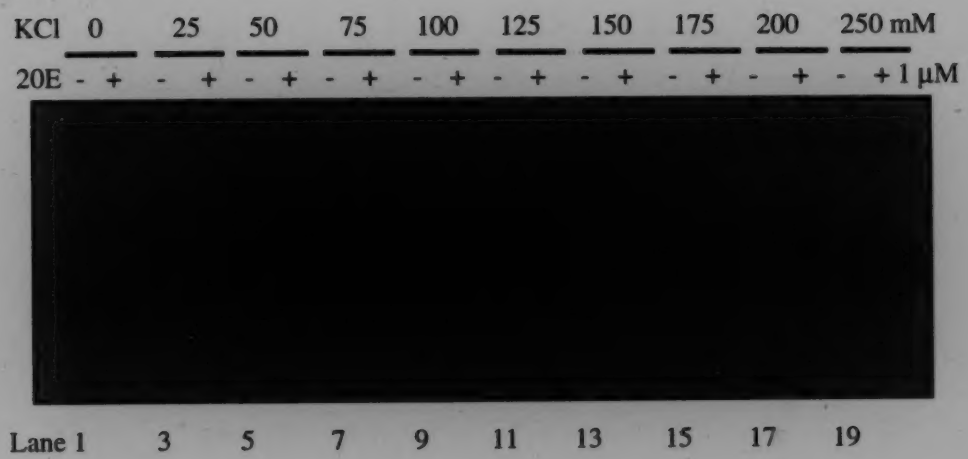
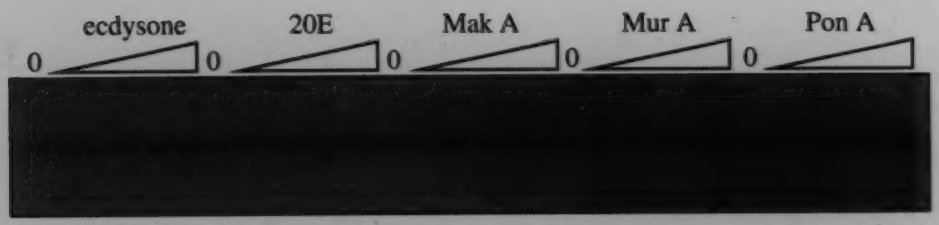
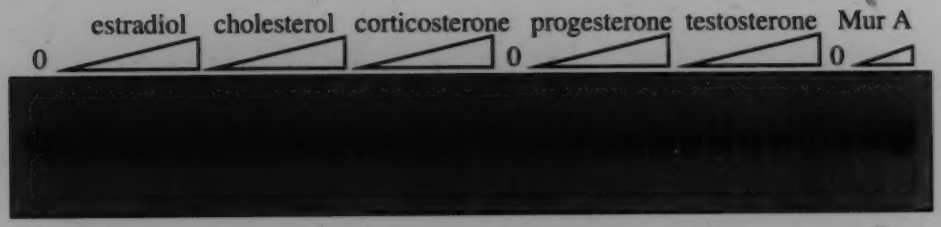

KCl 0 25 50 75 100 125 150 175 200 250 mM
20E - + - + - + - + - + - + - + - + - + - + 1 µM
Lane 1 3 5 7 9 11 13 15 17 19

Figure 3.4 Effect of ecdysteroids and vertebrate steroids on the binding of tick EcR to [^{32}P]hsp27 EcRE (only the shifted bands are shown). Tissue extract was incubated with ~1 nM [^{32}P]hsp27 EcRE in the presence of increasing amounts (0, 0.1, 1, 10, 100, 1000 nM) of the ecdysteroids (A) or (0, 0.02, 0.2, 2, 20, 200 μM) vertebrate steroids (B; 1 and 100 nM Mur A was run as a positive control on the same gel) for 60 min at 22°C. The wedges denote the lanes of increasing concentration. The incubation was resolved on a 4% polyacrylamide gel for 2 h at 22°C.

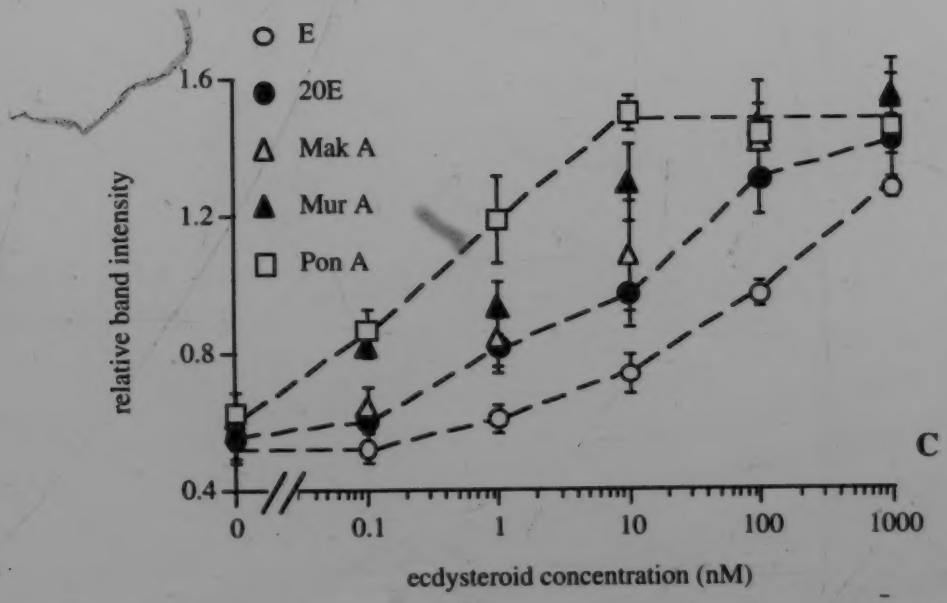
(C) Relative band intensity obtained from the PhosphorImager™ analyzer for the ecdysteroid data (n = 5). Data points are joined by dotted lines for Pon A, 20E and ecdysone.


ecdysone
20E
Mak A
Mur A
Pon A
0
0
0
0
0

A


estradiol
cholesterol
corticosterone
progesterone
testosterone
Mur A
0
0
0

B


E
20E
Mak A
Mur A
Pon A
relative band intensity
1.6
1.2
0.8
0.4
0
0.1
1
10
100
1000
ecdysteroid concentration (nM)

C

Figure 3.5 Western blot using mAbs against *Drosophila* EcR and USP. Salivary gland tissue extract (10 μg protein) was resolved by 7.5% SDS-PAGE. The gel was transferred onto a PVDF membrane which was subsequently incubated with 1:10 *Drosophila* mAb against EcR (AG10.2) and 1:100 *Drosophila* mAb against USP (AB11). The band signal was intensified by biotin-streptavidin and visualized by alkaline phosphatase and NBT-BCIP (see methods). There was no cross-reaction between the two mAbs. Molecular weight markers are indicated on the left. The three EcR bands are 90.5, 87.3 and 84 kDa (the faint band), and the two major USP bands are 50.3 and 47.1 kDa.

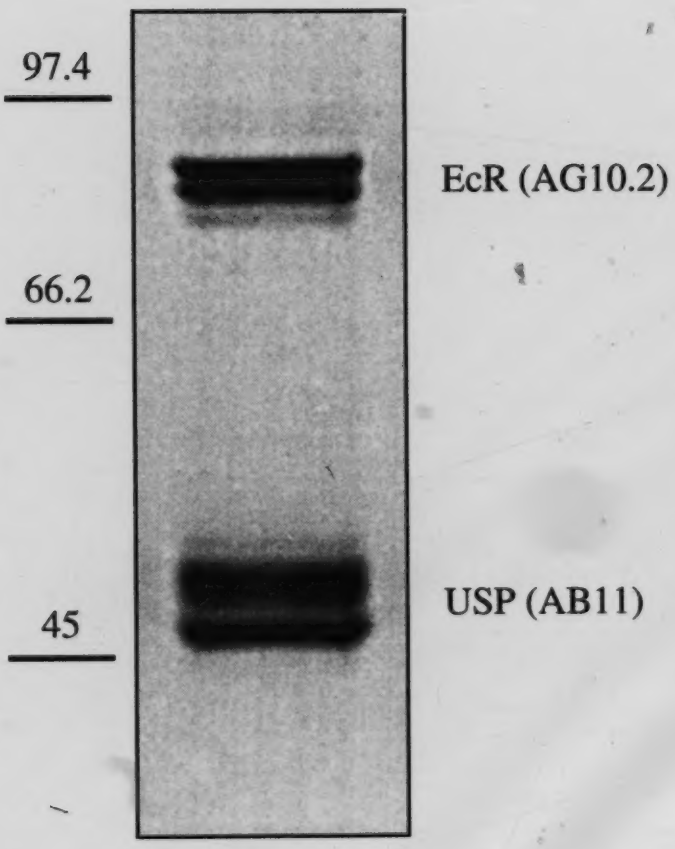

97.4
66.2
45
EcR (AG10.2)
USP (AB11)

Figure 3.6 Gel mobility supershift assay with *Drosophila* mAbs. The tissue extract was incubated with [^{32}P]hsp27 EcRE and 1 µl *Drosophila* mAb against USP (AB11), or antibodies against EcR isoform common regions (AG10.2, AC12.4, AG9.2, DDA2.7, JG6.2, IID9.6) for 60 min at 22°C. The mixture was resolved on a 4% polyacrylamide gel. The asterisk denotes the shifted protein-hsp27 EcRE complex band, and the arrow denotes the supershifted band.

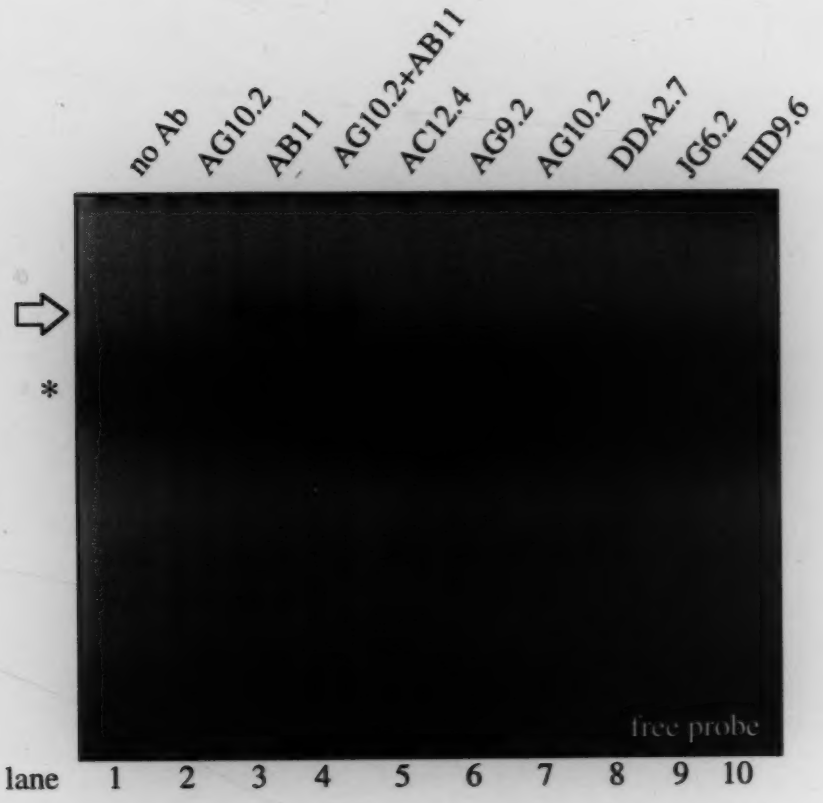
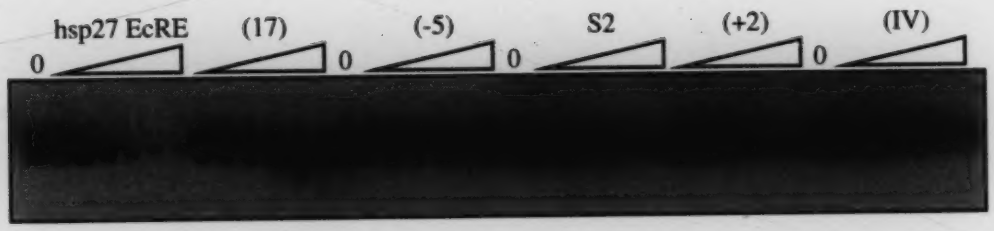
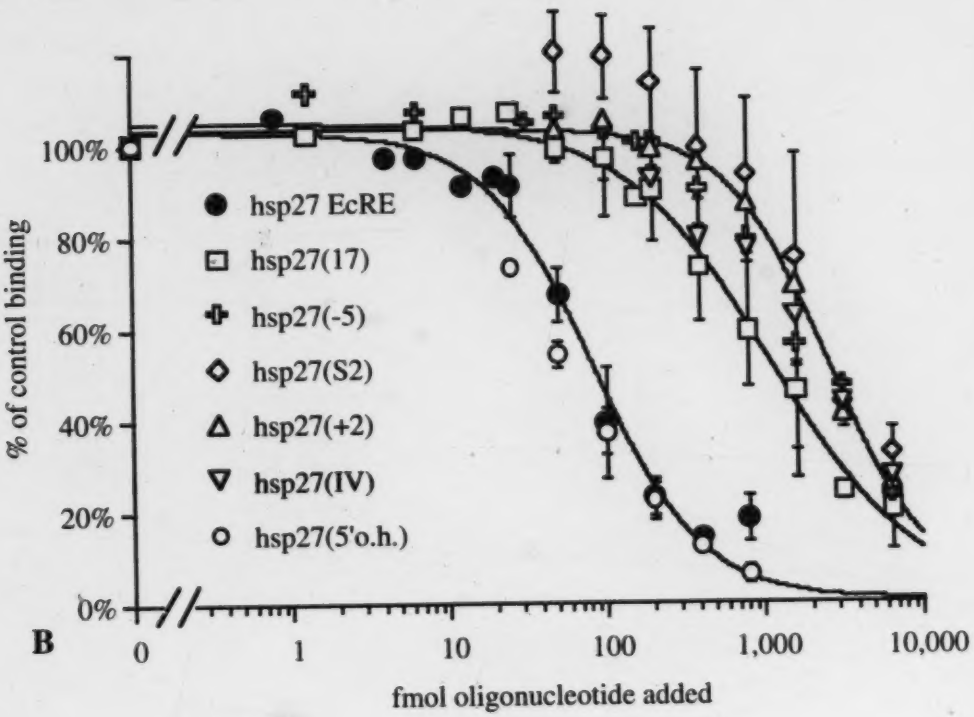

no Ab
AG10.2
AB11
AG10.2+AB11
AC12.4
AG9.2
AG10.2
DDA2.7
JG6.2
IID9.6
*
free probe
lane 1 2 3 4 5 6 7 8 9 10

Figure 3.7 (A) Competitive gel mobility shift assay between [^{32}P]hsp27 EcRE and unlabeled oligonucleotides (only the shifted bands are shown). Tissue extract was incubated with [^{32}P]hsp27 EcRE (5-10 fmol) in the presence of increasing amounts (0 - 6400 fmol) of synthesized oligonucleotides (see Table 3.1 for sequences) for 60 min at 22°C. The wedges denote the lanes of increasing amounts of the unlabeled oligonucleotides. The mixture was resolved on a 4% polyacrylamide gel.
(B) Relative binding is plotted (n = 2 - 6). Control binding in the absence of unlabeled oligonucleotide was defined as 100%. The curves for hsp27 EcRE, hsp27(17) and hsp27(+2) only are plotted (Deltagraph™ software). IC_{50} values calculated from the curves are shown in Table 3.2.

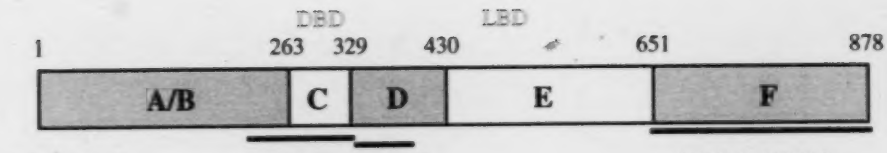

hsp27 EcRE
(17)
(-5)
S2
(+2)
(IV)
0
0
0
0
0

A


100%
80%
60%
40%
20%
0%
% of control binding
hsp27 EcRE
hsp27(17)
hsp27(-5)
hsp27(S2)
hsp27(+2)
hsp27(IV)
hsp27(5'o.h.)
0
1
10
100
1,000
10,000
fmol oligonucleotide added

B

Figure 3.8 Schematic representation of *D. melanogaster* EcR B1 isoform and epitope locations of various antibodies against *Drosophila* EcR (modified from Talbot *et al.*, 1993). The mAbs raised against EcR DBD are EEC11.1, AG9.2 and JG6.2. The mAbs raised against the "D" or "hinge" region are DDA2.7, IID9.6 and AAA5.4. The mAbs raised against the "F" region are AC12.4 and AG10.2, which is the region immediately following the LBD. Percent identity of EcR sequences are taken from Fujiwara *et al.* (1995) based on the results from *D. melanogaster* (Koelle *et al.*, 1991), *Aedes aegypti* (Cho *et al.*, 1995), *Chironomus tentans* (Imhof *et al.*, 1993), *Bombyx mori* (Swevers *et al.*, 1995) and *Choristoneura fumiferana* (Kothapalli *et al.*, 1995).



EcR antibody	EEC11.1 AG9.2 JG6.2	DDA2.7 IID9.6 AAA5.4		AC12.4, AG10.2
% identity	23-80	95-100	34-76	70-88	<10-46
Length	59-263	66	91-109	220-225	19-227

Table 3.1 Synthetic hsp27 EcRE, mutated oligonucleotides and assigned names

nucleotide position	7 6 5 4 3 2 -1 **0** +1 2 3 4 5 6 7
hsp27	A GA CA AGGGTTCA **A** TGCACTTGTCCAT
hsp27 (17)	AGGGTTCA **A** TGCACTTG
hsp27 (-5)	A GA CA AGGcTTCA **A** TGCACTTGTCCAT
hsp27 (+2)	A GA CA AGGGTTCA **A** Tt CACTTGTCCAT
hsp27 (S2)	A GA CA AGGGTTCA**Ag**TGCACTTGTCCAT
hsp27 IV	A GA CA AGGGTTCA **g** cat ACTTGTCCAT
5'GA TCCA GA CA AGGGTTCA **A** TGCACTTGTCCATCG *(5'o.h.)*
GT CT GT TCCCAAGT **T** ACGTGAACAGGTAGCTTAA 5'

One strand of each oligonucleotide and two strands of hsp27 (5'o.h.) are shown and aligned. In each sequence, the spacer is highlighted in bold and the two half sites of the imperfect palindrome are underlined. Mutated nucleotides are indicated with lowercase letters and named according to the mutated nucleotide positions.

Table 3.2 IC_{50} of synthetic oligonucleotides in displacing [^{32}P]hsp27 EcRE from tick EcR/USP

oligonucleotide*	IC_{50} (fmol)¶	ratio§
hsp27 EcRE (5'o.h.)	60	1.00
hsp27 EcRE	83	1.38
hsp27 EcRE (17)	1104	18.3
hsp27 EcRE (-5)	2089	34.7
hsp27 EcRE (+2)	2211	36.7
hsp27 EcRE IV	2496	41.4
hsp27 EcRE (S2)	2607	43.3

* See table 3.1 for assigned named of synthetic oligonucleotides

¶ IC_{50} for displacing [^{32}P]hsp27 EcRE binding was calculated from the curves shown in figure 3.7B.

§ IC_{50} of oligonucleotide/IC_{50} of hsp27 EcRE (5'o.h.)

Table 3.3 Comparison between ecdysteroid affinity for EcR and ED_{50} on EcR-DNA binding

Ecdysteroid	IC_{50} (nM)*	ED_{50} (nM)¶	IC_{50}/ED_{50}
ponasterone A	1.7	1	1.7
muristerone A	4	5	0.8
makisterone A	28	20	1.4
20-hydroxyecdysone	48	25	1.9
ecdysone	658	170	3.9

* Half maximal concentration of ecdysteroids needed to displace [^{3}H]Pon A binding from tick EcR (taken from Mao *et al* ., 1995)

¶ Half maximal concentration of ecdysteroids needed to enhance tick EcR/USP binding to hsp27 EcRE (calculated from figure 3.4C)

Table 3.4 Summary of molecular masses (kDa) of EcR and USP proteins among various arthropods determined by western blot analysis or deduced from amino acid sequences*

Species	EcR proteins	USP proteins
A. hebraeum (Acari)	90.5, 87.3	50.3, 47.1
D. melanogaster (Diptera)	105 (94), 80 [1]	54, 48 [2]
Aedes aegypti (Diptera)	74.6 [3]	54, 51.3 [4]
Bombyx mori (Lepidoptera)	68.1/70 [5]	50 [6]
Manduca sexta (Lepidoptera)	62.8, 63.6 [7]	52, 46 [8]
Choristoneura fumiferana (Lepidoptera)	67 [9]	
Anthonomus grandis (Coleoptera)	70 [10]	50 - 55 [10]

* Numbers underlined are molecular masses deduced from the amino acid sequences

[1] Koelle et al., 1991; Talbot et al., 1993
[2] Henrich et al., 1994
[3] Cho et al., 1995
[4] Kapitskaya et al., 1996
[5] Swevers et al., 1995
[6] Tzertzinis et al., 1994
[7] Fujiwara et al., 1995; Jindra et al., 1996
[8] Jindra et al., 1997
[9] Kothapalli et al., 1995
[10] Dhadialla and Tzertzinis, 1997

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Chapter 4

Profiles of the Ecdysteroid Hormone and its Receptor in the Salivary Gland of the Female Ixodid Tick, *Amblyomma hebraeum* †

Introduction

The feeding period of female ticks (Acari: Ixodidae) is divided into three phases: (i) a 'preparatory phase' (about 1-2 days), during which the feeding lesion is established, (ii) a 'slow phase' (lasting about 7-8 days), during which tick weight increases about 10-fold over the unfed weight, and (iii) a 'rapid phase' (lasting about 1 day), during which tick weight increases a further 10-fold (Balashov, 1972). At about the transition between the slow and rapid phases of engorgement there is a marked change in a number of behaviors (reviewed by Kaufman and Lomas, 1996). Because those behavioral changes are linked more specifically to achieved weight rather than to duration of feeding, it has been called the 'critical weight' (Harris and Kaufman, 1984; Lindsay and Kaufman, 1988; Kaufman and Lomas, 1996). We shall see shortly that some of the hormonal and ecdysteroid receptor (EcR) changes recorded here are also associated with the critical weight.

The salivary glands of female ixodid ticks perform several vital functions which facilitate tick feeding and achieve osmoregulation (reviewed by Kaufman, 1989; Sauer *et al.*, 1995). One major function is to secrete excess fluid from the blood meal back to the host; as a result, the blood meal is concentrated and the hemolymph volume regulated.

Although salivary glands of unfed female *Dermacentor andersoni* can secrete very little fluid *in vitro*, secretory competence *in vitro* increases almost 60-fold as feeding progresses (Kaufman, 1976). This physiological change is mirrored by a remarkable ultrastructural development of the fluid secretory cells (Fawcett *et al*; 1981a, b). I do not know what controls this development during the early feeding stage, although some

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† Manuscript submitted for publication.

evidence suggests that a hemolymph-borne factor(s) is involved (Coons and Kaufman, 1988). After the female tick engorges, the salivary glands are resorbed, an event characterized by the appearance of autophagic vacuoles (Harris and Kaufman, 1981). This autolytic process is triggered by an ecdysteroid hormone (Harris and Kaufman, 1985; Kaufman, 1991).

There is considerable evidence for the existence of a tick EcR: (i) A protein of the salivary gland binds to [^{3}H]ponasterone A (Pon A) in a manner similar to that observed for several insects and crustaceans (Mao *et al.*, 1995). I have demonstrated (ii) the specific Pon A binding-protein binds to hsp27 EcRE in a sequence-specific fashion; this binding is enhanced by a number of physiologically active ecdysteroids but is unaffected by vertebrate steroids; (iii) monoclonal antibodies (mAb) against *Drosophila* EcR and *ultraspiracle* protein (USP) recognize counterparts in tick salivary gland extracts by western blot analysis; and (iv) the mAb against USP retards the tick EcR-hsp27 EcRE complex on a gel mobility shift assay (Chapter 3).

In order to study the action of the ecdysteroid hormone at the molecular level, I monitored EcR/USP proteins in the salivary glands during a complete feeding cycle by western blot analysis. I also characterized the system by ligand- and DNA-binding assays in order to explore the functional state of the receptor, and compared these measures to the fluctuating titers of hemolymph ecdysteroid hormone.

Materials and Methods

Ticks

All stages of the African tick, *Amblyomma hebraeum* Koch, were reared in the laboratory on rabbits within a cloth-covered foam rubber area according to the method of Kaufman and Phillips (1973). The colony was held in darkness at 26°C and relative humidity > 95%. Adult males and females were fed together because mating occurs on the

host. Unfed female ticks weighed 28.4 ± 4.7 mg (mean ± SD, n = 100). Engorged females weighed 1592 ± 469 mg (mean ± SD, n = 127). Virgin female ticks were raised as follows: Males were enclosed in a cheese-cloth sac before adding them to the cloth-covered arena; this did not interfere with their attachment or release of aggregation-attachment pheromones, but did prevent transfer of spermatophores. Females were added to the cloth-covered arena the next day and forcibly removed at certain weight ranges. Smears of the seminal receptacle were routinely checked for the absence of spermatozoa.

Wet weight, dry weight, protein content and fluid secretory rate of the salivary glands from various feeding stages

Salivary glands from various feeding stages were dissected out in 1.2% NaCl. The glands were blotted gently to remove excess fluid and the wet weight recorded to the nearest 10 µg. The glands were then dried overnight at 60°C and dry weight recorded. Protein content of fresh glands homogenized in 1.2% NaCl was measured (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as a standard.

Fluid secretory rate was measured according to the method of Harris and Kaufman (1984). In brief, salivary glands were dissected out and wet weight recorded to the nearest 10 µg. Following incubation for 10 min in a modified TC medium 199 (Gibco) containing 10 µM dopamine (to stimulate fluid secretion), wet weight was recorded again. The weight difference (mg/gland/10 min) was the measure used for fluid secretory competence.

Radioimmunoassay (RIA) for measuring hemolymph ecdysteroids

Pooled hemolymph samples (5 - 40 µl total) from female ticks at various feeding/post-engorgement stages were collected via a lesion on the dorsal cuticle and mixed with 10 volumes of 100% methanol. The samples were stored at -15°C for RIA according to the method of Kaufman (1990). The ecdysone antiserum (ecdysone-22-succinylthyroglobulin) was a gift from Dr. L. I. Gilbert, University of North Carolina. The

ligand was α-[23,24-^{3}H(N)] ecdysone (54 Ci/mmol; Dupont NEN) and unlabeled 20-hydroxyecdysone (20E; Simes, Italy) was the standard. Because of the cross-reactivity of the antiserum among numerous side-chain modified ecdysteroids (Warren and Gilbert, 1986), our RIA data are expressed as '20E-equivalents'.

Ligand binding assay

The method was that of Mao *et al.*, (1995) with the following modifications. Pooled salivary glands (10 to 64 per sample depending on feeding stage of the ticks) were held in TEG-PMSF buffer (20 mM Tris, 1.5 mM EDTA, 10% Glycerol, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.5 at 22°C) for 10 min, and homogenized using a Brinkmann Polytron PT-10 (three bursts of 10 s with a 15 s intervening cooling period). The homogenate was centrifuged at 2,000 *g* for 10 min. The pellet was set aside while the supernatant was subjected to 100,000 *g* for 60 min at 4°C to produce a cytosolic fraction. The 2,000 *g* pellet was then washed in TEG-PMSF buffer twice, resuspended and incubated for 30 min in TEG-PMSF buffer containing 0.6 M KCl. The subsequent supernatant was dialyzed (MWCO: 10-14 kDa) against 2,000 volumes of TEG-PMSF buffer for 1 h to remove KCl and centrifuged at 13,000 *g* for 5 min; the resulting supernatant was designated the 'nuclear extract'. The tissue extracts were incubated with [^{3}H]Pon A (170 Ci/mmol, NEN Dupont) in the presence or absence of 1,000-fold excess of 20E for 4 h at 22°C. The incubation was terminated by adding dextran-coated charcoal at 4°C and radioactivity of the supernatant counted.

Scatchard analysis was used to estimate Bmax and Kd. Because specific Pon A binding was detected in the nuclear extract of our receptor preparation, the 'total EcR' was calculated by summing Bmax in both cytosol and nuclear extract for each salivary gland preparation. I do not know whether EcR in the cytosol fraction is an artifact of the extraction procedure, or reflects a natural sub-cellular distribution of the receptor. However, preliminary results from an immunohistochemical study suggest that the EcR is

located primarily in the nuclei (manuscript in preparation).

Gel mobility shift assay using Drosophila hsp27 EcRE as the probe

Synthetic *Drosophila* hsp27 EcRE and its complementary strand (imperfect palindrome is underlined and the spacer nucleotide highlighted in bold font, see below) were made on an Applied Biosystems 391 DNA Synthesizer PCR-MATE according to the manufacturer's instruction. The annealed probe was end-labeled with [γ-^{32}P]ATP (4,500 Ci/mmol, ICN), reconstituted in Tris-EDTA buffer (pH 7.6) and stored at 4°C (See Chapter 3 for further details).

5'GATCCAGACAAGGGTTC**A**ATGCACTTGTCCATCG
3'GTCTGTTCCCAAG**T**TACGTGAACAGGTAGCTTAA

Salivary glands from mated and virgin ticks at various feeding stages were homogenized in HEGD buffer (40 mM HEPES, pH 7.6, 1.5 mM EDTA, 10% glycerol, 5 mM dithiothreitol) and centrifuged at 70,000 *g* for 45 min. Protein concentration of the supernatant was determined by the method of Bradford (1976) using BSA as a standard. The tissue extract (10 µg protein) was incubated with ~20 fmol [^{32}P]hsp27 EcRE in the presence of 2 µg poly[dI-dC]•poly[dI-dC] (Sigma) for 1 h at 22°C. KCl (1.5 M) in HEGD buffer was added to make the final K^+-concentration of 75 mM. The binding mixture was resolved on a 4% pre-run (100 volts for 90 min at 22°C) polyacrylamide gel in 0.5X TBE buffer at 140 volts at 22°C. The gels were dried at 80°C for 1 h and exposed to X-ray film (X-OMAT™-AR, Kodak Scientific Imaging Film, New York) at -70°C. Relative band intensity was estimated by PhosphorImager™ analyzer and quantitated by ImageQuant™ software on a IBM computer.

Profiles of tick EcR and USP-like proteins revealed by western blot analysis

The salivary gland tissue extracts (see tissue preparation method for gel mobility shift assay) from mated and virgin ticks at various feeding stages were boiled with 2X

sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromphenol blue, and 10% ß-mercaptoethanol added just before use) for 5 min and resolved (10 µg total protein per lane) on a 7.5% SDS-polyacrylamide mini-gel (BioRad) at 90 volts. The gel was transferred to a PVDF membrane (BioRad) at 100 volts for 60 min and the latter was blocked with 5% skimmed milk/2% BSA in TTBS buffer (30 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.5) overnight at 4°C. The membrane was then blotted with *Drosophila* mAbs against EcR (1:10 AG10.2; obtained from Dr. P. Hurban, Stanford University School of Medicine; Talbot *et al.,* 1993) and USP (1:50 AB11; obtained from Dr. F. C. Kafatos, European Molecular Biology Laboratory, Heidelberg, Germany; Khoury Christianson *et al.,* 1992) at 22°C for 1 h. The PVDF membrane was subsequently incubated with biotinylated goat anti-mouse antibody (Sigma) and then with streptavidin conjugated alkaline phosphatase (BioRad). The protein bands were visualized by BCIP (5-bromo-4-chloro-3-indoylphosphate p-toluidine salt) and NBT (p-nitro blue tetrazolium chloride, BioRad).

Quantitative data of relative band intensity of EcR and USP were obtained from western blot analyses of five distinct preparations. The three bands of EcR and USP47-55 were combined and the band density was estimated by NIH Image software on a Macintosh computer.

Statistics

Data are reported as mean ± SEM (n). Statistical analysis (one-way ANOVA) was done with StatView 4.02™ software (Abacus Concepts, Inc.) on a Macintosh computer.

Results

Changes in wet, dry weight and protein content of the salivary glands throughout the feeding period.

During the slow phase of feeding, when salivary glands undergo enormous growth (Kaufman, 1976), the wet weight of the salivary glands increased 10-fold, dry weight 4.2-fold and protein content 4.4-fold over the corresponding values for unfed ticks (figure 4.1A). Peak values of these measures occurred at around the 'critical weight' (~300 mg, or 10X the unfed weight). Likewise, fluid secretory rate increased enormously (24-fold that of unfed glands, figure 4.1B) over the same period. All measures leveled off until the day of engorgement and fell dramatically during the first 4 days post-engorgement — the period of salivary gland autolysis.

The time course of mating during a normal feeding cycle is presented in table 4.1. The incidence of mating increased during the slow phase of feeding such that 83% of the ticks had mated by the time they reached 100 mg. Virtually all ticks had mated prior to achieving the 'critical weight' (97% of 100-200 mg of tick weight).

Profiles of hemolymph and salivary gland 20E-equivalents and specific Pon A binding activity throughout the feeding and early post-engorgement period

The hemolymph titer of 20E-equivalents in unfed ticks was 14.9 ± 1.2 pg/µl (n = 20, figure 4.2A). There was no significant change in hemolymph ecdysteroid titer in ticks up to 100-200 mg ($p = 0.92$, 0.38, 0.14 and 0.21 for <50 mg, 50-75 mg, 75-100 mg and 100-200 mg, respectively, compared to unfed). At around the critical weight there occurred a significant rise, peaking at 52 ± 4 pg/µl (n = 38) in ticks 500-700 mg ($p < 0.001$). Hemolymph 20E-equivalents fell back to 22 ± 1.5 pg/µl (n = 39) on the day of engorgement. As already reported several times (Connat *et al.*, 1985; Kaufman, 1991), there was a larger rise in the post-engorgement period, in this case reaching 742 ± 100

pg/µl (n = 5) by day 6 (figure 4.2A).

In figure 4.2B, the ecdysteroid content of salivary gland homogenates increased steadily during the slow phase of feeding and peaked at almost 10-fold that of unfed ticks (13 ± 1.3 pg 20E-equivalents/SG; n = 6 for unfed to 114 ± 11 pg 20E-equivalents/SG; n = 15 for 200-500 mg ticks). The ecdysteroid content of the tissue remained at this high level throughout the rapid phase of feeding and the period of salivary gland degeneration.

There was no detectable specific Pon A binding in the salivary glands of unfed ticks (figure 4.2C). A dramatic appearance of specific Pon A binding was observed within four days after the onset of feeding (820 ± 276 fmol/mg protein, n = 3, in ticks weighing below 50 mg). After this initial rise, Pon A binding plateaued throughout most of the feeding period, beginning to decline only towards the end. The decline continued through the early post-engorgement stage, and then seemed to rise slightly when salivary gland degeneration was advanced (day 6 post-engorgement).

The binding affinity between Pon A and tick EcR was calculated by Scatchard analysis (figure 4.2B, dashed bars). For this analysis, data were pooled as follows: (i) slow phase of feeding (ticks weighing below 100 mg), (ii) rapid phase of feeding (ticks weighing > 100 mg) and (iii) ticks between days 2-6 post-engorgement. The highest affinity for Pon A binding occurred in the second group (Kd = 1.2 ± 0.1 nM, n = 25). This affinity was significantly greater than that of tissue taken from ticks in the slow phase of feeding (Kd = 1.8 ± 0.3 nM, n = 21, $p < 0.05$) and that of tissue taken from post-engorged ticks (Kd = 2.3 ± 0.3 nM, n = 16, $p < 0.001$). Although Kd change (from 1.1-2.3 nM) of tick EcR for Pon A binding is modest significant, chages in affinity for 20E should be from ~ 50 nM to 115 nM (calculated from 48 nM; Mao *et al.*, 1995) theoretically. The affinity renge seems to be physiologically significant considering hemolymph 20E-equivalents were from 15 - 750 pg/µl, or ~ 30 - 100 nM during feeding (figure 4.2 A).

Profiles of tick EcR/USP binding to Drosophila hsp27 EcRE

I have demonstrated that tick EcR/USP binds to *Drosophila* hsp27 EcRE in a sequence-specific manner and that ecdysteroids enhance the binding as measured by a gel mobility shift assay (Chapter 3). Those experiments pertained to ticks weighing 200-700 mg. In figure 4.3, I show equivalent results of hsp27 EcRE binding for salivary gland extracts taken throughout the feeding cycle. There was no shifted band with tissue extract of salivary glands from unfed ticks. A faint band (5-pointed star in figure 4.3A) was seen in the salivary gland of ticks below 100 mg (in some replicates, this band was not seen). A shifted band (asterisk), slightly higher than the previous one, appeared when ticks exceeded 100 mg, and reached the highest intensity during the rapid phase of feeding. The band intensity decreased dramatically on days 0-2 post-engorgement, and no apparent band was present by days 4 to 6 post-engorgement. By adding a 50-fold excess of unlabeled hsp27 EcRE to the incubation medium of the tissue extract from 200-400 mg ticks, the shifted band was no longer seen (last lane of figure 4.3A).

In figure 4.3B is plotted the relative band intensity as a function of various feeding stages from 7 replicates of the experiment shown in figure 4.3A. The first significant appearance of specific DNA binding occurred in ticks exceeding 100 mg. DNA-binding increased significantly once again in tissue from ticks 400-600 mg; this coincides with the hemolymph ecdysteroid peak shown in figure 4.2A. The binding then decreased towards the end of feeding and dropped to low level in the post-engorgement period ($p < 0.005$).

Profiles of EcR and USP-like proteins revealed by western blot

In order to see profiles of EcR/USP-like proteins in the salivary glands, I screened the tissue extracts from various feeding stages by western blot analysis using *Drosophila* mAb AG10.2 and AB11 for EcR and USP respectively (figure 4.4A). In general, AG10.2 detected three protein bands with molecular masses of 90.5 ± 0.8, 87.3 ± 0.8 and 84 ± 0.8 kDa ($n = 18$). Four major bands recognized by AB11 were 52.6 ± 0.5, 50.3 ± 0.5, $47.1 \pm$

0.5 and 43.7 ± 0.7 kDa (n = 6 - 17), with a faint band of 55 ± 0.7 kDa (n = 2) appearing occasionally after the critical weight. Henceforth these proteins are named according to their size as EcR91, 87, 84 and USP55, 53, 50, 47, 44.

There were no EcR bands seen in the salivary glands of unfed ticks (figure 4.4A). Three EcR bands appeared after the onset of feeding and all of them intensified during the rapid phase of feeding The band intensity dropped significantly on days 4-6 post-engorgement.

The profile of USP proteins was more complicated (figure 4.4A). One, or occasionally two, USP bands were detected in the salivary glands of unfed ticks by AB11 which did not seem to co-migrate with any USP bands seen subsequently. USP44, USP47 and USP50 appeared in the salivary glands after the onset of feeding, and then USP44 disappeared when ticks exceeded 100 mg. USP47, USP50 and USP53 intensified during the rapid phase of feeding and reached the highest intensity (along with the transient appearance of USP55) in the salivary glands of ticks weighing 400-600 mg. USP53 and USP50 were sustained until day 2 post-engorgement. whereas USP47 remained through day 6.

Relative band intensity from 5 replicates of the western blot illustrated in figure 4.4A is shown in figure 4.4B. Basically, both EcR and USP proteins increased gradually during the slow phase of feeding and plateaued thereafter. The band intensity dropped following engorgement and decreased dramatically on days 4-6 post-engorgement.

EcR and USP profile from the salivary glands of virgin and mated ticks

In order to see if mating influences EcR/USP expression, I tested salivary gland extracts (by western blot) from virgin and mated ticks weighing above 100 mg, a time by which most females (that have been exposed to males) will have mated (table 4.1). There were no qualitative differences in banding pattern between virgin and mated ticks (figure 4.5). However, the salivary glands of mated ticks contained more EcR than those of

weight-matched virgins at the three weight ranges tested. Also, the USP55 band seemed to be more abundant in the salivary glands of mated ticks.

DNA binding properties of EcR/USP from salivary glands of virgin and mated ticks

DNA binding of the salivary gland tissue extracts from virgin and mated ticks slightly below (100-200 mg) and above (300-400 mg) the critical weight was tested using hsp27 EcRE as a probe in a gel mobility shift assay (figure 4.6). EcR/USP from the salivary glands of virgin ticks formed a binding complex with the same shifted mobility as that of their mated counterparts. However, there was significantly more EcR/USP-hsp27 EcRE complex formed in the salivary glands of mated ticks.

Discussion

The purpose of this study was to correlate hemolymph ecdysteroid titer to EcR/USP levels in the salivary glands throughout the feeding cycle of adult female ticks. This is a first step in understanding at the molecular level how the ecdysteroid hormone initiates salivary gland degeneration.

Figure 4.7 summarized the profile data of the hormone and receptor in order to show relevant correlations. In unfed ticks, hemolymph ecdysteroid titer was low (15 pg/µl; figure 4.2A), and I could detect no EcR by western blot, ligand-binding or DNA-binding. Within a few days of feeding (ticks < 50 mg), the appearance of EcR bands (figure 4.4) coincided with a high level of ligand-binding (figure 4.2C), both of which were maintained through the end of feeding. At this early stage, however, absence of a major DNA-binding complex (figure 4.3) suggests that the receptor was not yet fully functional. If heterodimerization with a USP-like protein is necessary for DNA-binding as it is in other systems (Yao *et al.*, 1992; 1993; Thomas *et al.*, 1993; Swevers *et al.*, 1996), neither USP47 nor USP44 is likely to be the correct species. USP47 was present at all feeding stages, including unfed ticks, and was especially abundant in feeding ticks below 100 mg

(figure 4.4). USP44 was present only transiently during the early days of feeding, and disappeared before there could be any ecdysteroid-mediated salivary gland degeneration.

Most females (69-83%) mate during the slow phase of feeding (50-200 mg; table 4.1). It was only at the latter part of this period (100-200 mg) that specific DNA-binding became detectable; DNA-binding increased significantly around the first hemolymph ecdysteroid peak (figure 4.3). This period corresponded to a high level of two species of USP bands (USP50 and USP53). The EcR bands were also strong throughout this period and were maintained until at least day 2 post-engorgement (figure 4.4).

During the last day or so of feeding there was a significant peak in hemolymph ecdysteroid titer (52 pg/µl; figure 4.2A), a period characterized by maximal levels of DNA-binding and by the highest affinity of ligand-binding, indicating a fully functional receptor. In general, all the measurements taken in this study reached their highest level at around the time of the hormone peak.

It is only during the early post-engorgement period (days 1-4) that salivary gland degeneration manifests itself (Harris and Kaufman, 1984). According to our DNA-binding data, there was a reduction of functional receptor by day 0-2 post-engorgement (figure 4.3), accompanied by a decrease in USP53 and USP55. USP50 and USP53 virtually disappeared by days 4-6, and no DNA binding was detected (figure 4.3).

On the basis of the foregoing, I propose that the ligand binding site is associated with the EcR bands, but ligand-binding alone is not sufficient for a functional receptor. USP50 and/or USP53 are essential modulators of ecdysteroid receptor function; without them, DNA-binding, and presumably gene activation, do not occur.

Significance of the hormone peak during the rapid phase of engorgement

I am the first to report the occurrence of a significant peak of hemolymph ecdysteroid titer during the feeding period (figure 4.2A). This peak corresponded approximately with achieving the critical weight. Kaufman and Lomas (1996) reviewed

some physiological and behavioral changes that occur around the critical weight. Ticks forcibly removed from a host below the critical weight do not suffer salivary gland degeneration and do not lay eggs. Ticks forcibly removed from a host above the critical weight, on the other hand, usually refuse to reattach and resume feeding, even if given the opportunity; their salivary glands degenerate within 4 days. One thing that does seem clear is that the large rise in hemolymph ecdysteroid titer occurring after day 2 post-engorgement (figure 4.2A) is not the trigger for salivary gland degeneration because autolysis is well under way before that time (Lomas *et al.*, 1998).

Lomas *et al.* (1998) suggested that the salivary gland tissue concentration of ecdysteroid is higher than the hemolymph titer during the first 48 h post-engorgement (when salivary gland degeneration is taking place). Could the small hemolymph ecdysteroid peak lead to the higher tissue hormone level, and thus be the trigger for salivary gland autolysis? The peak level observed (52 pg/µl) is above the threshold for triggering salivary gland degeneration *in vitro* (20-30 pg/µl; Harris and Kaufman, 1985; Lomas and Kaufman, 1992a). It is also above the Kd range of 20E for this receptor (~23 pg/µl, Mao *et al.*, 1995). Salivary gland ecdysteroid content persisted after the hemolymph peak and was maintained at a high level in spite of a drop in hemolymph titer on the day of detachment (figure 4.2B). Furthermore, I also observed an increased intensity of DNA-binding to EcR/USP at around the hormone peak. I have shown that exogenous ecdysteroids enhance binding between tick EcR/USP and hsp27 EcRE (figure 3.5). I am thus tempted to speculate that the small ecdysteroid peak may be an important signal in a manner analogous to the "commitment peak" of ecdysteroid in larval Lepidoptera (Nijhout, 1994) and *Drosophila* (Riddiford, 1993) which triggers the behavioral changes leading to metamorphosis.

However, this signal seems to be reversible or not sufficient in ticks. Harris and Kaufman (1985) and Lomas *et al.* (1998) demonstrated that salivary glands of recently engorged ticks (and thus presumably already exposed to the rapid-phase ecdysteroid peak)

fail to degenerate *in vitro* in the absence of exogenous ecdysteroid. A possible explanation for this is that, without exogenous ecdysteroid, endogenous hormone diffuses out of the glands held *in vitro* before it has triggered mRNA synthesis. This explanation is supported by the observation that similar glands do continue degenerating *in vitro,* in the absence of exogenous ecdysteroid, if they are dissected out of the tick 1 or 2 days post-engorgement (Harris and Kaufman, 1985, Lomas *et al.*, 1998). Another potential signal in the salivary gland degeneration process is the act of detachment. Kaufman and Lomas (1996) have recently postulated, on the basis of circumstantial evidence, that the degeneration process does not begin in ticks above the critical weight until they disengage from the host.

A more mundane explanation to consider for the ecdysteroid peak during the rapid phase of feeding is that changes in fluid movement across the gut wall should influence the titer of any substance in the hemolymph. Thus hemolymph ecdysteroid titer at any moment will reflect changes in rate of synthesis/release, uptake by tissue, metabolism, excretion and the balance between absorption of fluid from the gut and secretion of saliva. During the rapid phase of engorgement the rate of fluid absorption and salivary fluid secretion intensify greatly. An imbalance in the latter two processes might also explain (or at least contribute to) the changes in hemolymph ecdysteroid-titer observed at the end of feeding.

Mating influences expression of EcR and USP

Figures 4.5 and 4.6 demonstrate that the salivary glands of mated females contain more EcR, which bind to DNA more intensely than the EcR from weight-matched virgins. This suggests that 'male factor' (a protein in the spermatophore transferred from male to female ticks during copulation; Harris and Kaufman, 1984; Lomas and Kaufman, 1992a, b; Kaufman and Lomas, 1996) might affect EcR/USP expression besides hastening the appearance of ecdysteroids in mated ticks (Lomas and Kaufman, 1992a). It seems that lower EcR results in lower DNA binding capacity in large fed virgins. I do not yet know the physiological significance of this differential binding, because the glands of virgin and

mated females have similar sensitivities to 20E *in vitro* (Lomas and Kaufman, 1992a). The fact that 20E seems to up-regulate this EcR-system (figure 5.2, 5.3) further complicates the interpretation of these *in vitro* studies.

Figure 4.1 (A) Profile of wet weight (open squares, n = 10-32), dry weight (open triangles, n = 5-14) and protein content (closed triangles, n = 5-13) of salivary glands during the feeding and post-engorgement periods. The overall changes that occurred during the rapid phase of feeding are statistically significant ($p < 0.0001$ for all three parameters). CW, SG and SGD stand for critical weight, salivary gland and salivary gland degeneration, respectively. Tick weights are indicated on the abscissa for partially-fed ticks, and days post-engorgement are indicated for engorged ticks. SEM is indicated wherever it exceeds the dimension of the symbol.

(B) Profile of salivary gland secretory rate throughout the feeding cycle; n = 5-14. The secretory rates of the salivary glands from ticks during the rapid phase of feeding were significantly higher than those of ticks below 100 mg or those of ticks on days 2-4 post-engorgement ($p < 0.0001$). Secretory rate of salivary glands at 100-200 mg ticks was significantly higher than that of ticks below 100 mg ($p < 0.005$).

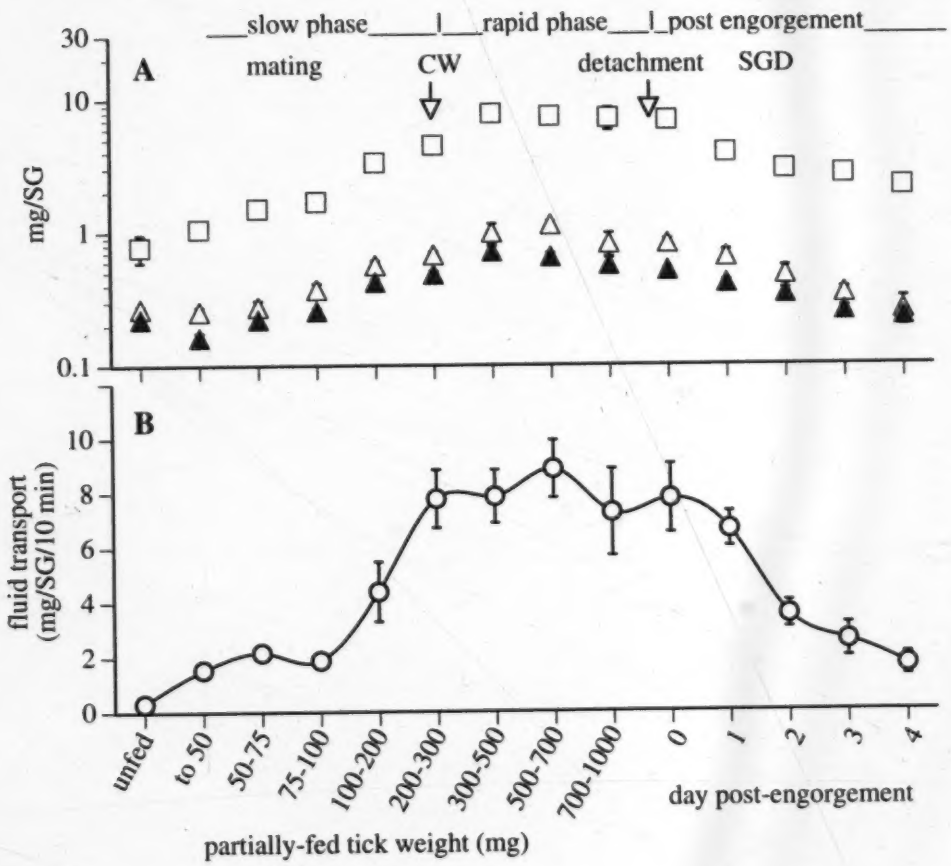

slow phase
rapid phase
post engorgement
A
mating
CW
detachment
SGD
mg/SG
30
10
1
0.1
B
fluid transport (mg/SG/10 min)
10
8
6
4
2
0
unfed
to 50
50-75
75-100
100-200
200-300
300-500
500-700
700-1000
0
1
2
3
4
day post-engorgement
partially-fed tick weight (mg)

Figure 4.2 (A) Profile of hemolymph titer of 20E-equivalents throughout feeding and up to 6 days post-engorgement in female ticks. N = 19-73 except day 6 post-engorgement where n = 5. The SEM does not exceed the dimension of any symbol. Statistical significance is as follows: hemolymph concentrations in ticks up to 200 mg and on days 0-3 post-engorgement were significantly lower than the concentration in ticks weighing 500-700 mg ($p < 0.001$). The hemolymph concentrations on days 4-6 post-engorgement were significantly higher ($p < 0.001$) than at any other stage.

(B) Profile of 20E-equivalent content (pg/salivary gland) throughout feeding and up to 6 days post-engorgement (n = 5-15). The peak salivary gland 20E-equivalent content from the ticks weighing above 200 mg was significantly higher ($p < 0.01$) than that of all stages below 100 mg.

(C) Profile of specific [^{3}H]Pon A binding to tissue extract as calculated by Scatchard analysis.(n = 3 - 6 except unfed where n = 2). Statistical analysis was not performed due to the small sample sizes. However, there was no detectable specific binding component in either of the two cytosolic or nuclear extracts from unfed ticks. The dashed bars represent Kd values calculated from Scatchard analysis for the data pooled into three groups: (1) feeding ticks below 100 mg (1.8 ± 0.3 nM, n = 21), (2) ticks greater than 100 mg and including day 0 post-engorgement (1.1 ± 0.1 nM, n = 25) and (3) days 2-6 post-engorgement (2.3 ± 0.3 nM, n = 16). Affinity for Pon A in the middle group was significantly greater (*) than that for the small ticks ($p < 0.05$) and the engorged ticks ($p < 0.001$).

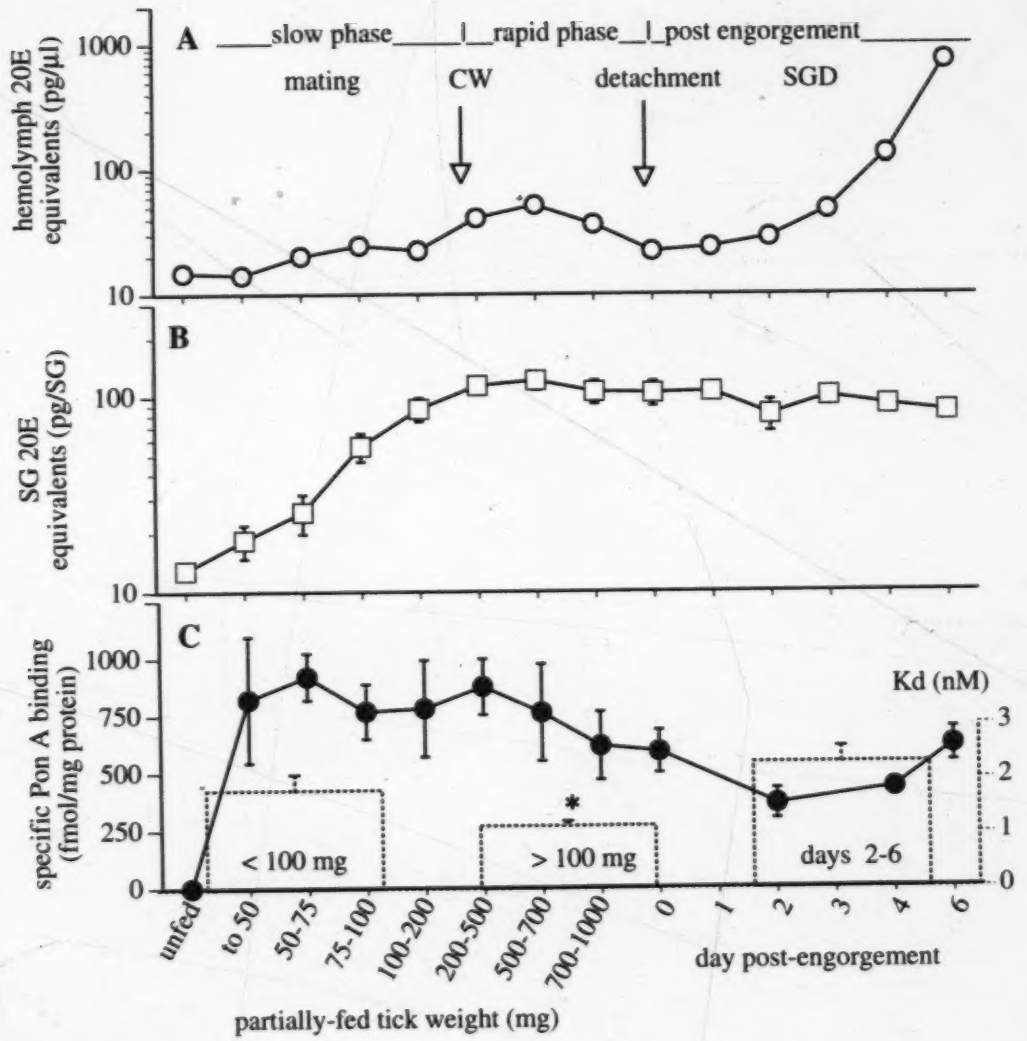
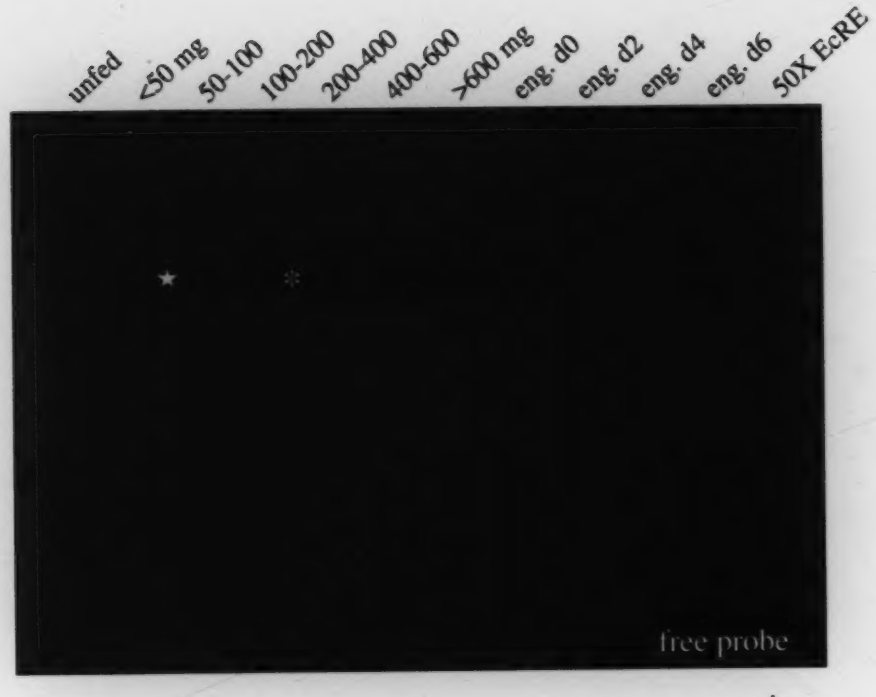
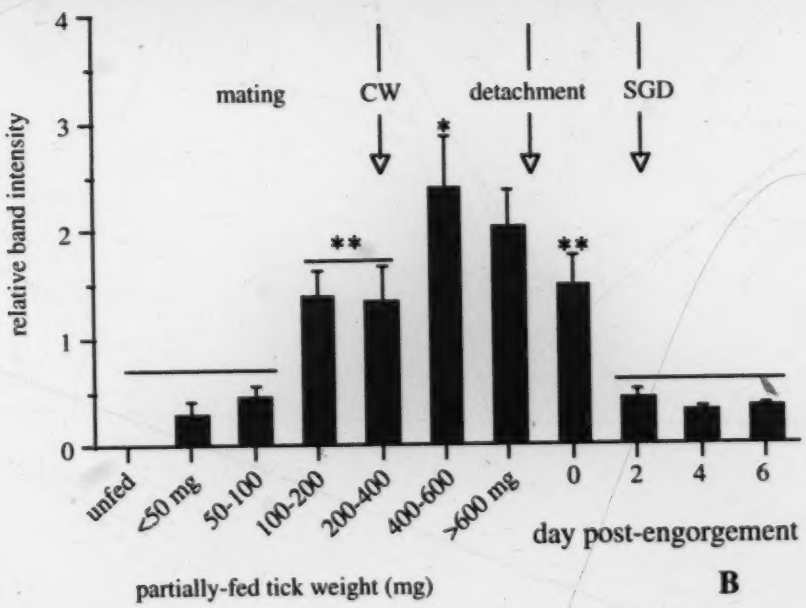

A
slow phase
rapid phase
post engorgement
mating
CW
detachment
SGD
hemolymph 20E equivalents (pg/µl)
1000
100
10
B
SG 20E equivalents (pg/SG)
100
10
C
specific Pon A binding (fmol/mg protein)
1000
750
500
250
0
Kd (nM)
3
2
1
0
*
< 100 mg
> 100 mg
days 2-6
unfed
to 50
50-75
75-100
100-200
200-500
500-700
700-1000
0
1
2
3
4
6
day post-engorgement
partially-fed tick weight (mg)

Figure 4.3 (A) DNA binding profile of salivary gland tissue extracts from ticks at various feeding stages. Tissue extracts (10 µg protein) were incubated with ~20 fmol [^{32}P]hsp27 EcRE and 2 µg poly[dI-dC]•poly[dI-dC] for 60 min at 22°C. The mixture was resolved on a native 4% acrylamide gel at 22°C and exposed to X-ray film. The major shifted band (asterisk) was not seen in the salivary glands of unfed ticks nor in those of ticks below 100 mg; in the latter, a faint band (5-pointed star) was seen only in some preparations. In the last lane, a 50-fold excess of unlabeled hsp27 EcRE was included in the tissue extract from 200-400 mg tick salivary glands, and demonstrates complete displacement of the [^{32}P]hsp27 EcRE.

(B) Relative intensity of the shifted bands measured by the PhosphorImager™ analyzer ($n = 6 - 8$). Intensity of the shifted bands in ticks of 400-600 mg was significantly higher than that of any previous stage and that of days 0-6 post-engorgement ($p < 0.01$). Intensity of the bands in ticks of 100-400 mg was significantly higher than that of any of the previous stages ($p < 0.01$). Intensity of band in ticks on day 0 post-engorgement was significantly higher than any of the later stages ($p < 0.005$).

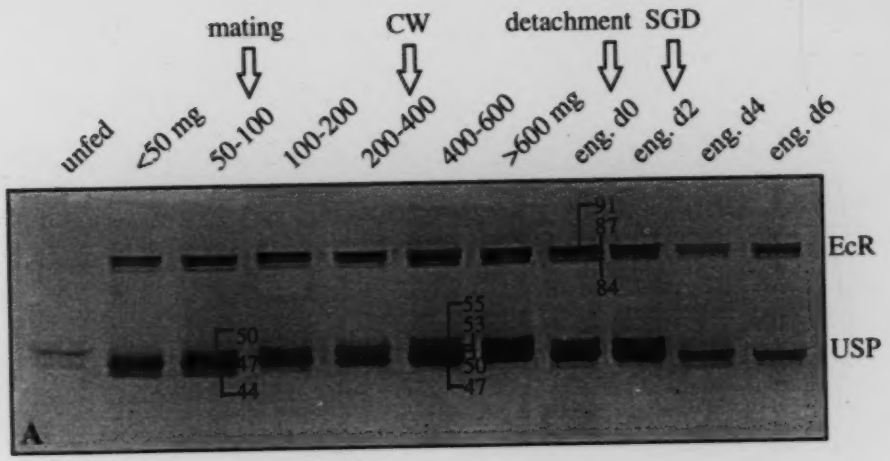

unfed
<50 mg
50-100
100-200
200-400
400-600
>600 mg
eng. d0
eng. d2
eng. d4
eng. d6
50X EcRE
free probe

A


relative band intensity
mating
CW
detachment
SGD
unfed
<50 mg
50-100
100-200
200-400
400-600
>600 mg
0
2
4
6
day post-engorgement
partially-fed tick weight (mg)

B

Figure 4.4 (A) Western blot of tick EcR and USP proteins throughout feeding and post-engorgement. Tissue extract (10 μg of total protein) from salivary glands of various stages were resolved on 7.5% SDS-PAGE. *Drosophila* mAb AG10.2 and AB11 were used to immunoblot the transferred PVDF membrane to detect EcR and USP bands. Three EcR bands (top) are 90.5 ± 0.76, 87.3 ± 0.78 and 84 ± 0.8 kDa (n = 18) respectively. There were no detectable EcR bands in the tissue extract of unfed tick salivary glands. USP proteins appeared as four major bands with molecular masses at 52.6 ± 0.54, 50.3 ± 0.48, 47.1 ± 0.47 and 43.7 ± 0.69 kDa (n = 6 - 17). There was also a transient band of 55 ± 0.7 kDa (n = 2) which appeared at the rapid phase of feeding. Although the EcR band density in this salivary gland preparation from the ticks at day 6 post-engorgement was higher than that of day 4 ticks, in other preparations. the band density at day 6 was either similar to or lower than that of day 4 ticks.
(B) Relative band intensity of EcR/USP throughout feeding cycle obtained from the quantitative data determined by NIH Image software on a Macintosh computer (n = 5). For this analysis, all EcR bands were pooled and USP bands (except USP44) were pooled. All bands increased in intensity during the feeding period and decreased in the post-engorgement period ($p < 0.05$, ANOVA).


mating
CW
detachment
SGD
unfed
<50 mg
50-100
100-200
200-400
400-600
>600 mg
eng. d0
eng. d2
eng. d4
eng. d6
EcR
USP
91
87
84
55
53
50
47
50
47
44
A

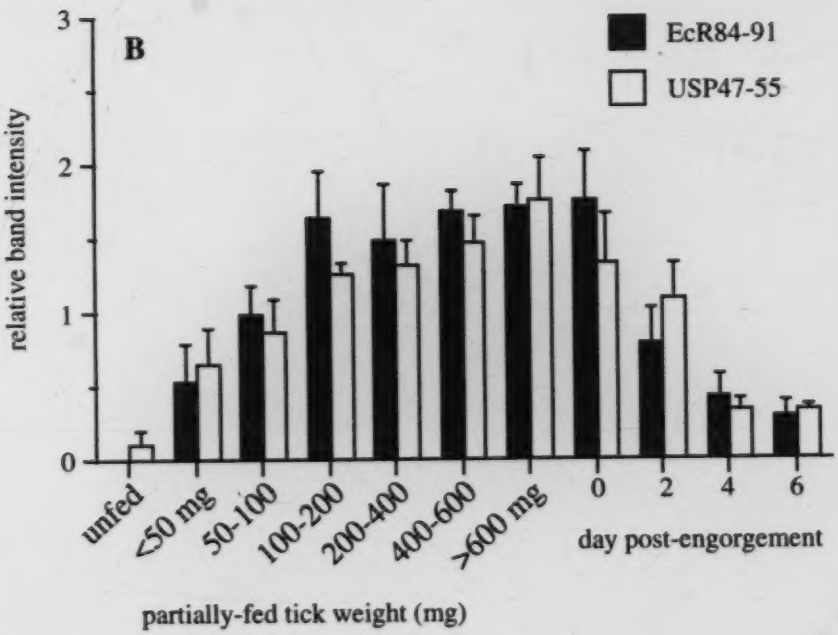

B
EcR84-91
USP47-55
relative band intensity
3
2
1
0
unfed
<50 mg
50-100
100-200
200-400
400-600
>600 mg
0
2
4
6
day post-engorgement
partially-fed tick weight (mg)

Figure 4.5 Western blot of tick EcR and USP from the salivary glands of virgin (V) and mated (M) ticks from three feeding stages. Tissue extracts (10 µg of total protein) were applied on a 7.5% SDS-PAGE and transferred onto a PVDF membrane. *Drosophila* antibodies AG10.2 and AB11 were used to immunoblot EcR and USP bands.

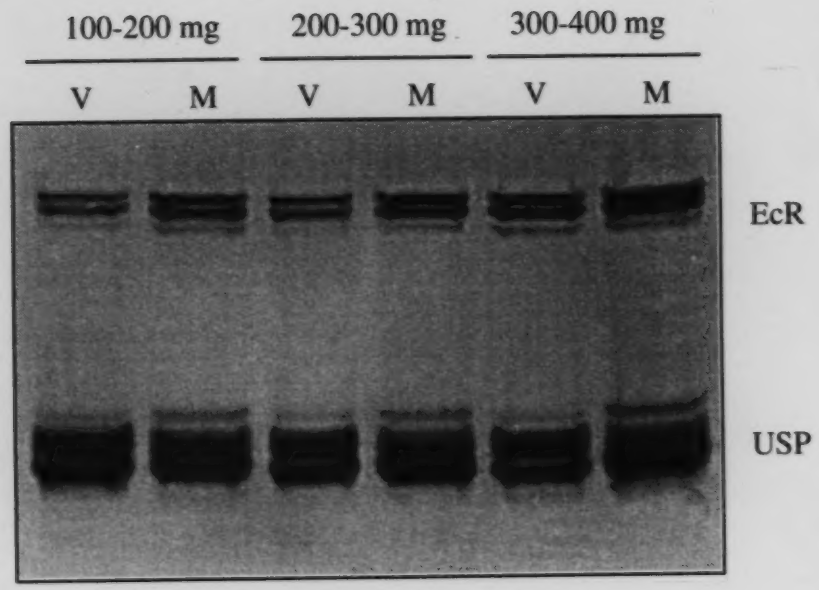

100-200 mg
200-300 mg
300-400 mg
V
M
V
M
V
M
EcR
USP

Figure 4.6 DNA binding profile of salivary gland tissue extracts of virgin (V) and mated (M) ticks from two feeding stages. Tissue extracts (10 µg of total protein) were incubated with ~20 fmol [^{32}P]hsp27 EcRE and 2 µg poly[dI-dC]•poly[dI-dC] for 60 min at 22°C. The mixture was resolved on a native 4% acrylamide gel at 22°C and exposed to an X-ray film. The shifted band is indicated by the arrow.

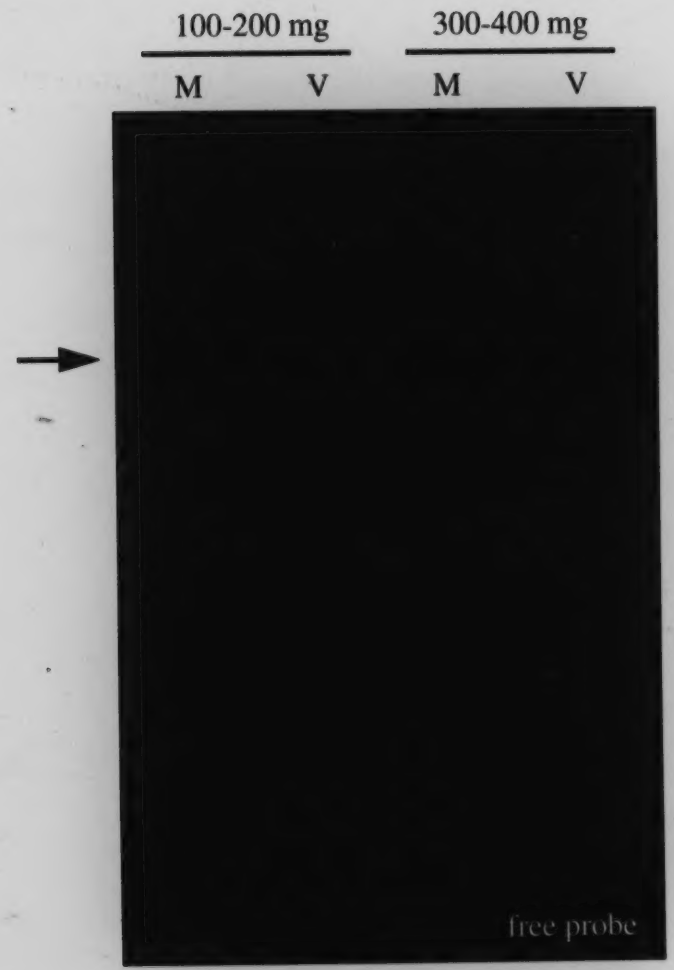

100-200 mg
300-400 mg
M
V
M
V
free probe

Figure 4.7 Comparison of the profiles among hemolymph 20E-equivalents, EcR and USP in the salivary gland and their DNA binding profile throughout feeding and post-engorgement. Data were taken from figure 4.2 A, 4.3 B and 4.4 B, respectively, and only mean values are plotted. Band intensity of USP50-53 were chosen and plotted in USP profile figure.

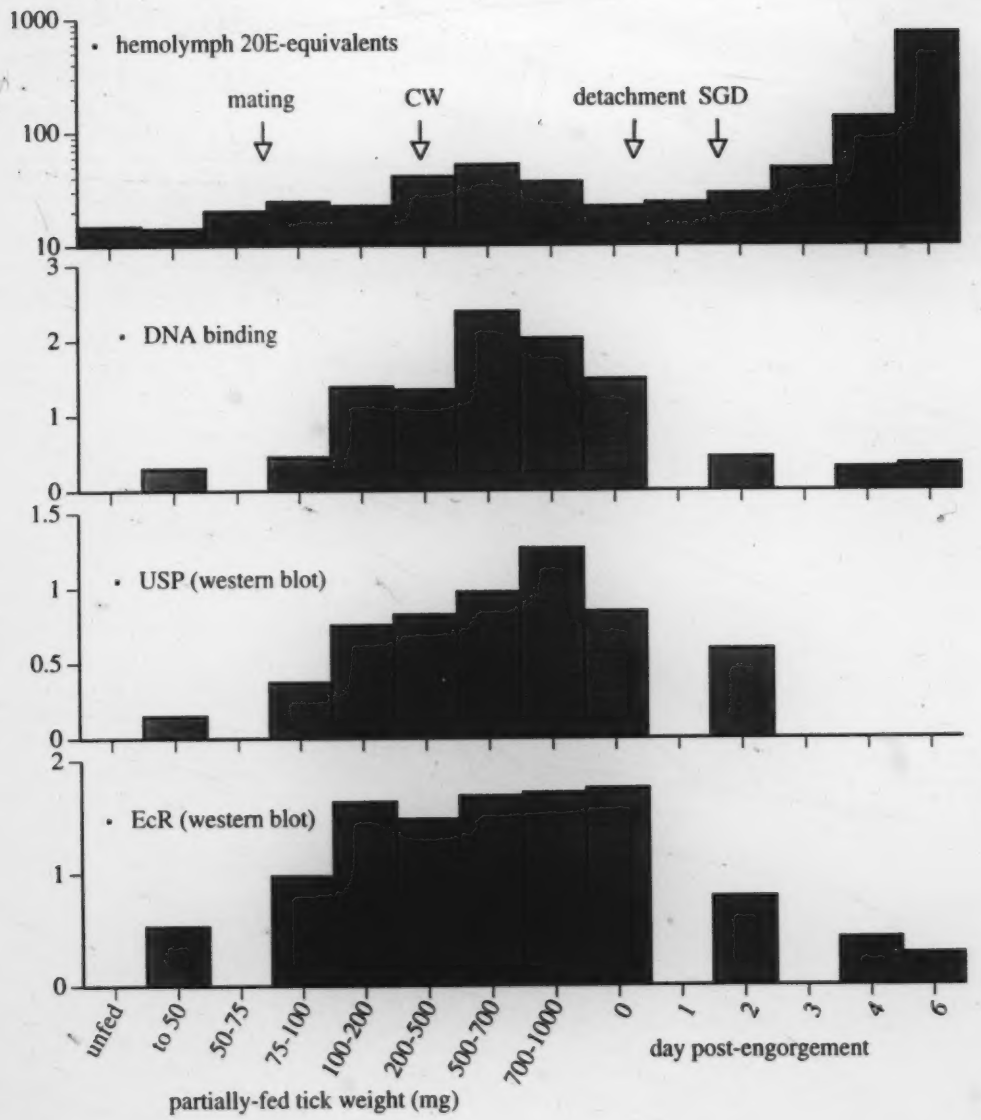

1000
100
10
• hemolymph 20E-equivalents
mating
CW
detachment
SGD
3
2
1
0
• DNA binding
1.5
1
0.5
0
• USP (western blot)
2
1
0
• EcR (western blot)
unfed
to 50
50-75
75-100
100-200
200-500
500-700
700-1000
0
1
2
3
4
6
day post-engorgement
partially-fed tick weight (mg)

Table 4.1 Progress of mating during the feeding cycle of *Amblyomma hebraeum*

tick weight	feeding peroid†	#mated/#examined	percent
< 40 mg	2	0/11	0%
40-50 mg	4	2/14	14%
50-75 mg	5	18/26	69%
75-100 mg	7	24/29	83%
100-200 mg	8	30/31	97%
200-300 mg	10	50/52	96%
300-500 mg	12	31/32	97%
> 500 mg	12	32/32	100%

† Approximate days to reach the tick weight range indicated.

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Chapter 5

General Discussion and Conclusions

General Conclusions

Prior to the experiments described in this thesis, it was known that ticks, like all arthropods, use an ecdysteroid hormone for several functions, including moulting, salivary gland degeneration and possibly vitellogenesis (table 1.2). The specificity of the ecdysteroid effect on the salivary gland of *Amblyomma hebraeum* strongly suggested that it was a receptor-mediated response, but until very recently, there were no reports in the literature dealing with the EcR of ticks. This thesis has established:

(1) There is a protein in the salivary gland which binds to [^{3}H]Pon A with high affinity and limited capacity. The basic biochemical and pharmacological properties of the binding protein correspond to those expected for an EcR (chapter 2).

(2) Use of a specific *Drosophila* EcRE probe enabled me to demonstrate that the DNA-binding properties of the tick EcR are similar to those established for some insects (chapter 3).

(3) There are several species of EcR and a USP-like protein in the tick salivary gland, and the profile of these bands changes significantly throughout the feeding cycle (chapter 4).

(4) Although the evidence that an ecdysteroid hormone triggers salivary gland generation has been accumulating over the years, a major anomaly has been that salivary gland degeneration begins in engorged females before hemolymph ecdysteroid titer increases significantly. I have uncovered a possible solution to this anomaly: a significant ecdysteroid peak during the rapid phase of engorgement (which has not been observed previously) may be the hormonal signal which triggers salivary gland degeneration (chapter 4).

I do not have direct evidence to show that the small hormone peak does initiate apoptosis in the salivary gland, however, two strands of evidence suggest that this small peak might be the signal. Firstly, salivary glands from ticks above the critical weight (thus

exposed to the small peak) 24-48 h post-removal undergo degeneration spontaneously if the glands are set up in organ culture, whereas ones from ticks below the critical weight (not exposed) do not. Secondly, the large ecdysteroid peak which begins to rise on day 3 post-engorgement cannot be the trigger of apoptosis because salivary gland degeneration is well underway by 24 h post-engorgement (Harris and Kaufman, 1984; Lomas *et al.*, 1998). However, a definitive demonstration of the physiological importance of this peak will require the availability of an ecdysteroid synthesis inhibitor.

This hormonal signal seems to be reversible. Harris and Kaufman (1985) and Lomas *et al* (1998) demonstrated that salivary glands of recently engorged ticks (and thus presumably already exposed to the small ecdysteroid peak) fail to degenerate *in vitro* in the absence of exogenous ecdysteroid. As already mentioned just above, the trigger to degenerate is irreversible within 48 h post-removal. Perhaps the process is analogous to other apoptosis systems in which programmed cell death involves gene activation and *de novo* mRNA and protein synthesis (Tata, 1996). For example, programmed motoneuron death triggered by ecdysteroid in *Manduca* abdominal ganglia is reduced dramatically when neurons are treated with actinomycin D or cycloheximide (Weeks *et al.*, 1993; Fahrbach *et al.*, 1994). This suggests that apoptotic genes belong to the 'late gene' category according to the Ashburner model (figure 10.1). The situation might be similar in ticks.

A possible role for interaction between neuronal and ecdysteroid signals on neuron death was also tested in *Manduca* (Fahrbach and Truman, 1987). Isolation of a pair of motoneurons in the third abdominal ganglion from the anterior nervous system rescued those cells from the apoptosis. In the latter system, the neural signals may act cooperatively with the ecdysteroid decline to specify the exact time for triggering the death of individual neurons (Fahrbach and Truman, 1987). Kaufman and Lomas (1996) hypothesized that the act of detachment of the tick from its host may constitute an important signal in salivary gland degeneration and ovarian development. If this hypothesis is correct (it has not yet been tested), the detachment signal is likely to be a neuronal one.

I propose the following model of ecdysteroid hormone action on salivary gland degeneration (figure 5.1). Mating accelerates feeding to the critical weight (Kaufman and Lomas, 1996). After ticks exceed the critical weight, stretch receptors in the abdominal dorso-ventral muscles send a signal to the synganglion through opisthosomal nerves (Harris and Kaufman, 1984). The synganglion releases a peptide to stimulate epidermal tissue to synthesize and release more ecdysteroid hormone (Lomas *et al.*, 1997). This produces the ecdysteroid peak observed during the rapid phase of feeding. Elevated ecdysteroid binds to EcR/USP-EcRE, which in turn activates early genes that determine the fate of the salivary gland (capable of apoptosis). However, a neuronal signal resulting from detachment is also required to complete the signal leading to the late gene activation and salivary gland degeneration.

Very recently, Guo *et al* (1997) have cloned a tick EcR from *Amblyomma americanum*. AamEcR shares 86-98% and 55-63% identity in DBD and LBD respectively with the insect EcRs, which suggests that it is an authentic EcR. The *ecr* gene of *A. americanum* encodes three isoforms, which is in agreement with the number of protein bands detected by *Drosophila* antibody in *A. hebraeum* (figure 4.4). However, the molecular masses from our western blot analysis (90.5, 87.3, 84 kDa) are different from the predicted sizes deduced from AamEcR mRNA sequences (62.7, 61.6, 48.4 kDa). These differences in size may be explained by post-translational modification of the protein and/or by whether the EcR possesses an F region. Guo *et al.* (1997) reported that all the three AamEcR isoforms lack F regions. Because AheEcR bands are recognized by AG10.2, an antibody raised against the F region of *Drosophila* EcR, it suggests that AheEcR possesses an F region, and this would account, at least in part, for the larger size of AheEcRs. Guo *et al.* (1997) also showed that AamEcR requires an AamRXR for *in vitro* transactivation in a mammalian cell line. This result agrees with my result demonstrating that a USP-like protein is involved in DNA-binding (figure 3.6). Two transcripts of

AamEcR increased steadily during feeding in the salivary gland of female ticks, another factor in good agreement with the results in chapter 4.

Future Directions:

EcR in the salivary gland is induced following the onset of feeding. I have preliminary results to show that a specific Pon A binding component can be induced by exposing salivary glands of unfed ticks to 20E *in vitro*. The induction is time- (minimum 3 h, figure 5.2) and dose-dependent (threshold concentration 10 pg/µl of 20E, figure 5.3). I have not yet confirmed whether the specific Pon A binding component is EcR by either western blot or hsp27 EcRE-binding assay.

A more accurate way to measure the induction of the *ecr* gene would be to monitor its mRNA (rather than protein) in the cultured salivary gland. Since a tick EcR has been cloned, finding a cDNA probe to monitor the induction of the *ecr* gene is now an exciting prospect.

A more reliable and sensitive method should be developed to detect the onset of salivary gland degeneration. Fluid secretion is the biological effect resulting from the cooperation of numerous enzymes involved in the signal transduction pathway of salivary gland secretion (e.g., Na^+/K^+-ATPase: Kaufman *et al.*, 1976 and Rutti *et al.*, 1980; adenylate cyclase: Schmidt *et al.*, 1982 and Kaufman, 1986). A decrease in Na^+/K^+-ATPase or adenylate cyclase is likely to be a late event in the apoptotic process. Some tests to monitor early gene activity would be more desirable.

Figure 5.1 Schematic drawing of a suggested model for ecdysteroid action on salivary gland degeneration. Profile of hemolymph ecdysteroid titer is taken from figure 4.2A.

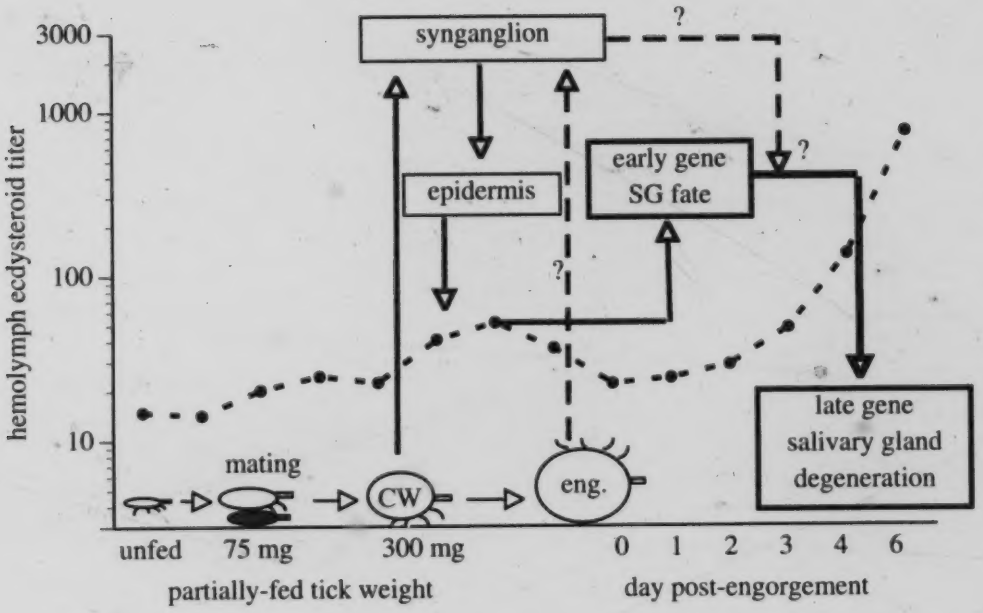

synganglion
?
epidermis
early gene
SG fate
?
?
late gene
salivary gland
degeneration
hemolymph ecdysteroid titer
3000
1000
100
10
mating
CW
eng.
unfed
75 mg
300 mg
0 1 2 3 4 6
partially-fed tick weight
day post-engorgement

Figure 5.2 Time course of specific Pon A binding induced by 20E in organ culture. Salivary glands (8) of unfed ticks were incubated in Grace's medium at 22°C with (closed circles) or without (open circles) 500 pg 20E/µl (n = 2 - 4). Incubation was stopped at the time indicated by freezing the tissue to -70°C. Specific Pon A binding was conducted by incubating the tissue extract with 10-20 nM [^{3}H]Pon A in the presence or absence of a 2,000-fold excess of 20E for 5 h at 22°C. The specific dpm was calculated as the difference between total binding and non-specific binding. The specific dpm ratio was calculated as the quotient of each specific dpm by the maximal dpm in that run. There was no specific binding of Pon A at 0 h. The maximum specific binding at 96 h was 3114 ± 196 dpm for 20E-treated (n = 3) compared to the control of 195 ± 87 DPM (2). Non-specific binding was 17 ± 2% of total binding.

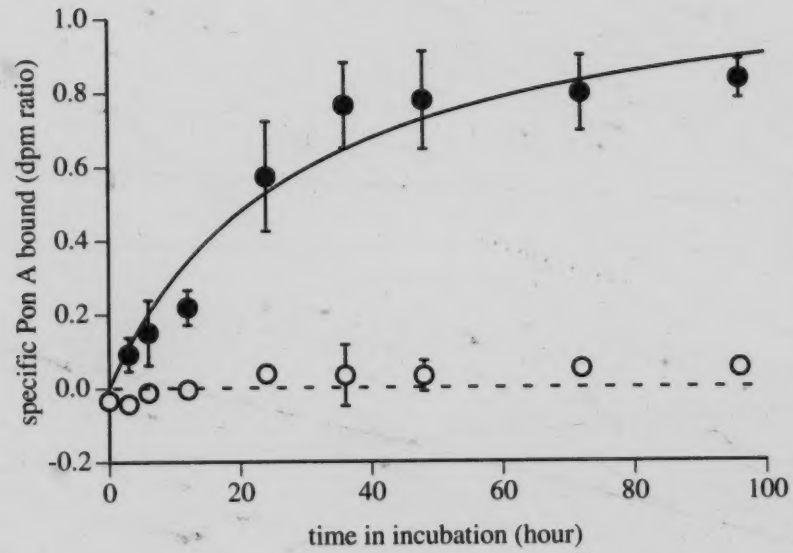
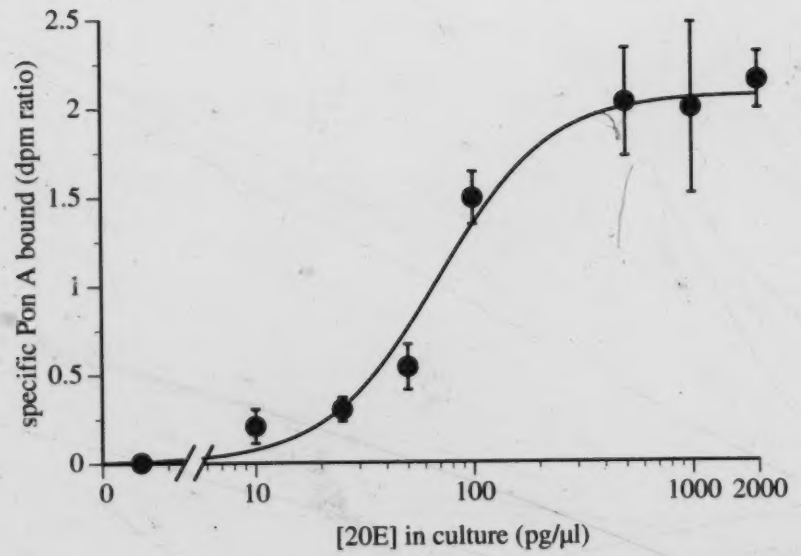

1.0
0.8
0.6
0.4
0.2
0.0
-0.2
specific Pon A bound (dpm ratio)
0
20
40
60
80
100
time in incubation (hour)

Figure 5.3 Dose-response curve of specific Pon A binding induced in cultured salivary glands from unfed ticks (n = 2 - 4). Salivary glands (8) of unfed ticks were incubated in Grace's medium with various concentrations of 20E for 30 h (see legend for figure 5.2). ED_{50} was calculated as 69 pg/µl based on the formula derived from the best-fit curve plotted by Deltagraph™ software on a Macintosh computer.


specific Pon A bound (dpm ratio)
2.5
2
1.5
1
0.5
0
0
10
100
1000 2000
[20E] in culture (pg/µl)

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Appendix: Sedimentation coefficients of tick EcR revealed by sucrose density gradient centrifugation

Before the western blot technique was developed for this study, receptor forms were investigated by sucrose density gradient centrifugation. Based on known studies on vertebrate steroid receptors, I had tested the followings factors in order to see if EcR displays different forms under different experimental conditions.

1. Is there a 'cytosol' and a 'nuclear' form of EcR?
2. Effect of KCl on 'cytosolic' EcR
3. Effect of hsp27 EcRE on tick EcR
4. Effect of molybdate on tick EcR
5. Effect of dithiothreitol on tick EcR

The method and modifications were as indicated in each legend. Non-specific binding was tested by incubating [^{3}H]Pon A with 200-1000 fold excess of 20E in each experiment. For all the results in this appendix, the specific [^{3}H]Pon A-binding peak was abolished by the excess 20E as what was shown in figure 2.7 (results not shown).

Two different EcR forms were detected in the 'cytosolic' (3.2S) and 'nuclear' (5.4S) fractions, respectively (figure A1a). Because the large nuclear EcR form was not seen consistently, I suspect that the large nuclear form was induced by KCl treatment. However, I do not know whether the large 'nuclear' form consisted of EcR/EcR homodimers or EcR/USP heterodimers. Results shown in figure A2 suggested that it is a heterodimer.

In order to see if KCl induced the large form of the 'nuclear' EcR, 0.4 M KCl was used to treat the cytosolic fraction and no large form was seen. This results seemed to suggest that there are large and small EcR forms present in different cellular compartments. Because a minimal KCl concentration was required for EcR/USP-EcRE binding tested by gel mobility shift assay (figure 3.3), it is likely that EcR/USP dimerization also requires a

certain ion strength in the buffer. After dialysis, the nuclear extract in this preparation (figure A1a) might have contained the right salt strength for dimerization. Whereas 0.4 M KCl used in the 'cytosolic' fraction in figure A1b might be too high for the formation of the dimer, based on the results shown in figure 3.3. Cytosolic EcR in a lower concentration of KCl (~ 0.1 M) needs to be tested in order to know whether it is a KCl effect.

By introducing *Drosophila* hsp27 EcRE to the Pon A-EcR incubation, a large form (6S) was induced in a dose-dependent manner (figure A2). Because tick EcR binds to hsp27 EcRE with USP (figure 3.6), it is likely that the large form consists of EcR and USP. Because I have not tested if the large forms ('nuclear' EcR and one induced by EcRE) co-migrate on the sucrose density gradient during a single run, whether they are the same components remains unknown.

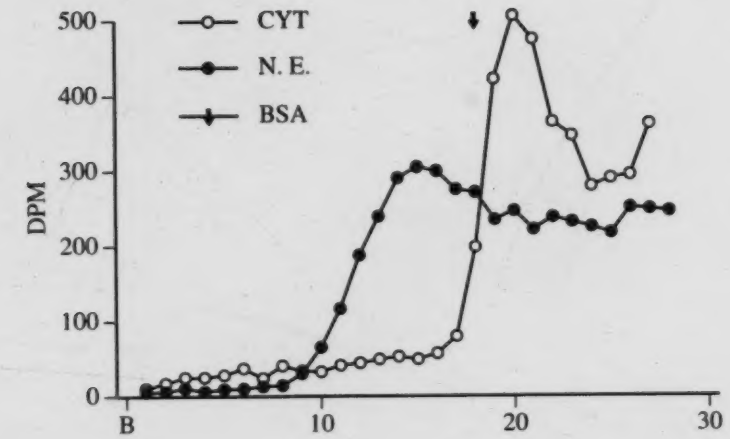
Molybdate has been shown to stabilize the large form of vertebrate steroid receptors i.e., the complex formed among heat shock proteins and the receptor (see reference to Leach *et al.,* 1979 in Chapter 2). In order to see if molybdate has a similar effect on tick EcR (i.e., whether the tick EcR associates with an analogue of heat shock protein), 10 mM molybdate salt was used throughout the experiment. Figure A3 showed that molybdate had no effect on tick EcR form in low reducing medium (≤ 0.5 mM dithiothreitol, DTT). However, it reduced the proportion of the large form (6S) apparently induced by 5 mM DTT (see below), which suggests that tick EcR might not associate with a chaperone protein.

By introducing various concentration of dithiothreitol to the buffers, a large 'cytosolic' form (6S) was induced in a dose-dependent manner (figure A4). Again, I do not know whether this large form co-migrates with any of the large forms mentioned above and whether it is an EcR/EcR homodimer or an EcR/USP heterodimer. We know that thiol-modifying reagents interrupted Pon A-EcR binding (figure 2.1), thus a reducing enviroment is essential, not only for ligand binding and but possibly also for EcR/USP dimerization. However, one unique feature for tick EcR is that EcR seems to be able to

bind to its ligand without assistance of USP (the small forms shown on sucrose density gradient), which disagrees with studies in *Drosophila*.

In conclusion, tick EcR seems to display different forms under different ion strength. A large 6S form, possibly EcR/USP heterodimer, can be induced by *Drosophila* hsp27 EcRE in a dose-dependent manner. Tick EcR tends to form a large 5.4S form under reducing conditions, and molybdate seems to reduce formation of the 5.4S form.

Figure A1. Sedimentation coefficients of cytosol and nuclear EcR tested by sucrose density gradient centrifugation. Salivary glands from large partially-fed ticks were homogenized in TE buffer with 0.5 mM PMSF and 0.3 M sucrose (TEPS). Cytosol (CYT) and nuclear fraction were separated by 1,000 *g*, 10 min centrifugation. Part of the cytosol was incubated with 0.4 M KCl for 30 min (**b**). The 1,000 *g* pellet was rinsed in TEPS buffer once and incubated with 0.6M KCl-TEDP buffer for 30 min. All the extracts were centrifuged at 100,000 *g* for 60 min and the supernatants saved. The nuclear extract was dialyzed against 2,000-fold TEP buffer for 4 h (MWCO: 10 kDa). The dialyzed nuclear extract (N.E.) was centrifuged on 12,000 *g* for 5 min to remove the precipitate. All the extracts were incubated with ~5 nM[^{3}H]Pon A ± 20E for 4 h and treated with dextran-treated charcoal (DCC). The supernatant (~80 µl) and [^{14}C]BSA (~2 µl. 4.35 S as a internal standard) were loaded on 10-40% (**a**) or 5-20% (**b**) sucrose density gradient.. The gradients were centrifuged for 13 h at 286,000 *g* at 3°C. The resulting solution was collected 9 drop-wise and radioactivity of each fraction measured. The 'cytosolic' fraction displayed 3.2 S whereas the 'nuclear' fraction 5.4 S.


CYT
N. E.
BSA
DPM
500
400
300
200
100
0
B
10
20
30

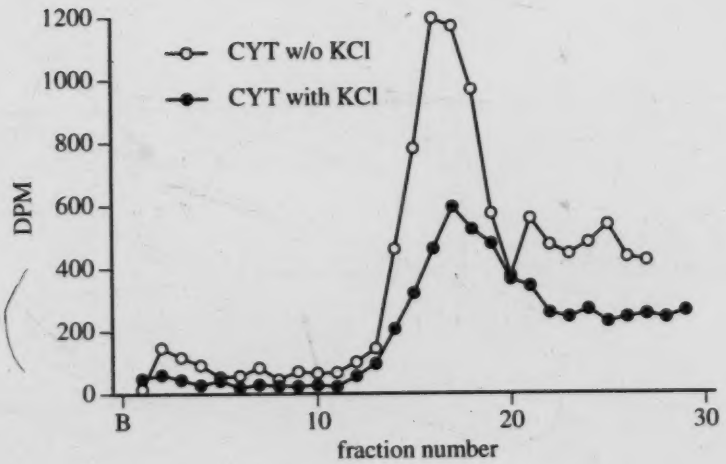

CYT w/o KCl
CYT with KCl
DPM
1200
1000
800
600
400
200
0
B
10
20
30
fraction number

Figure A2. Effect of double-stranded hsp27 EcRE on tick EcR. The large peak in (**a**) is magnified and replotted in (**b**). The 100,000 *g* supernatant of the salivary gland tissue extract in 40 mM HEPES buffer with 2 mM EDTA, 5 mM DTT and 0.08 M KCl was incubated with 5 nM [^{3}H]Pon A ± double-stranded hsp27 EcRE for 2 h at 23°C. After DCC treatment, the supernatant was loaded on a 10-40% sucrose density gradient and centrifuged at 286,000 *g* for 13 h. Ten drops were collected for each fraction and radioactivity of each fraction counted. The large form was 6 S and the small form 3.2 S.

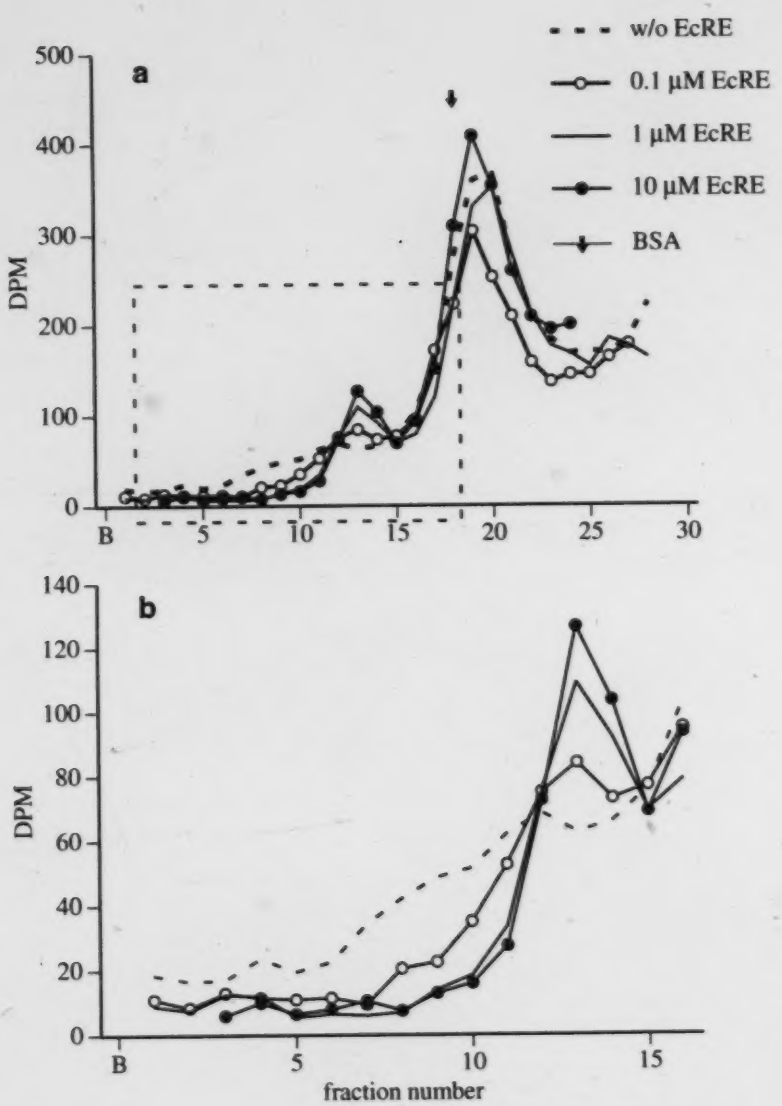

w/o EcRE
0.1 μM EcRE
1 μM EcRE
10 μM EcRE
BSA
a
b
DPM
DPM
fraction number
B
5
10
15
20
25
30

Figure A3. Effect of molybdata on EcR sedimentation coefficient. Salivary glands from large-partially fed ticks were homogenized in TE buffer ± 10 mM MoO4 with various amount of dithiothreitol (DTT: 0 mM in **a**, 0.5 mM in **b** or 5 mM in **c**). The homogenate was centrifuged at 100,000 *g* for 40 min and the supernatant was incubated with 5 nM ± 2000-fold 20E for 4 h at 23°C. After DCC treatment, the supernatant (80 µl) was layered on 10-40% sucrose density gradient (5-20% sucrose gradient in **a**). After centrifuging at 286,000 *g* (55,000 rpm in SW55 rotor), 3°C for 14 h, the gradient was fractionated in 9 drop-wise (7 drop-wise in **a**) and radioactivity of each fraction counted. The large form was about 6S and the small form 3.2S.

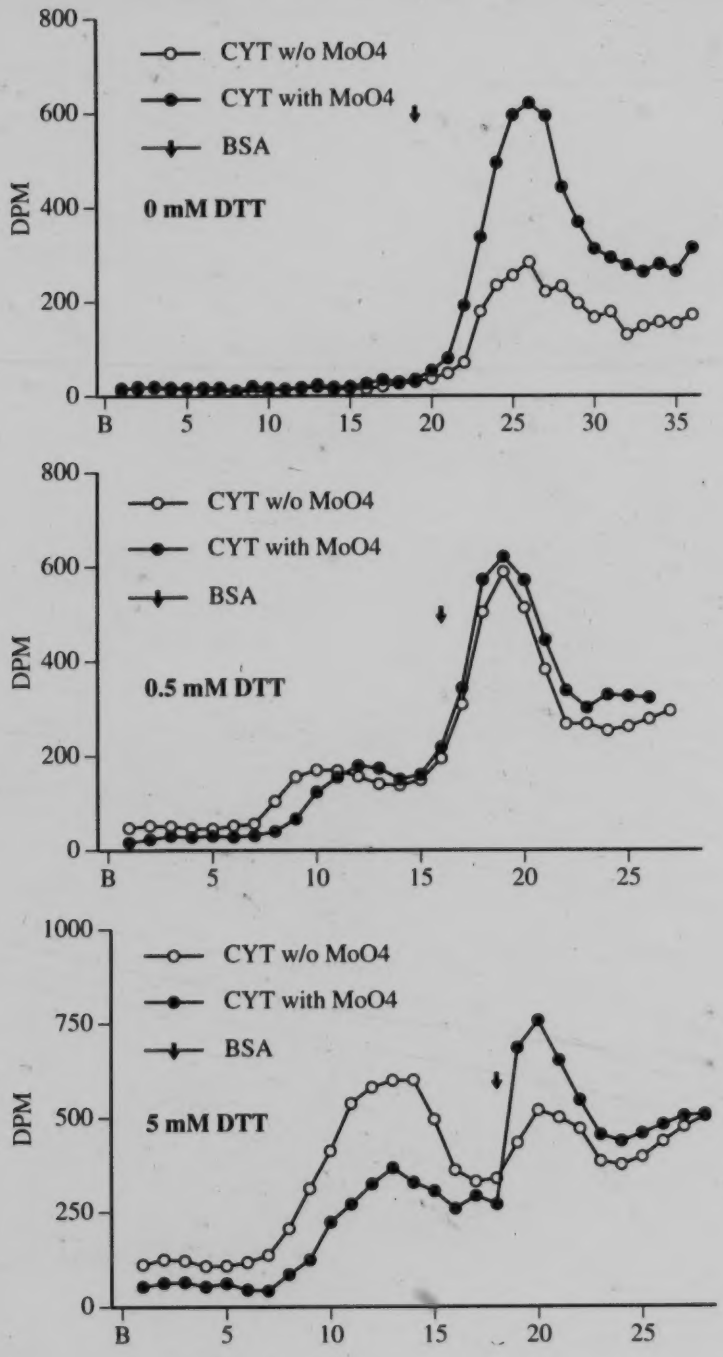
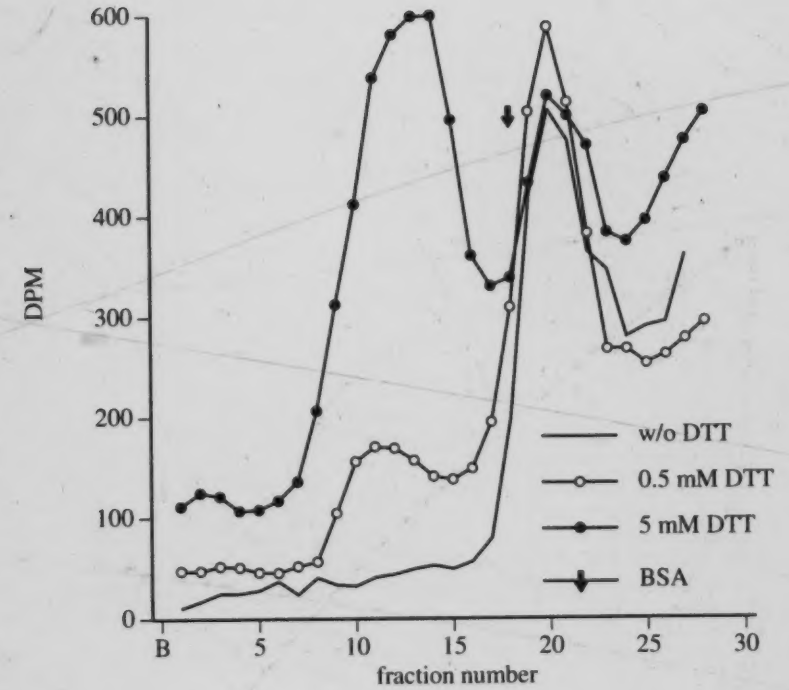

800
600
400
200
0
DPM
CYT w/o MoO4
CYT with MoO4
BSA
0 mM DTT
B
5
10
15
20
25
30
35
800
600
400
200
0
DPM
CYT w/o MoO4
CYT with MoO4
BSA
0.5 mM DTT
B
5
10
15
20
25
1000
750
500
250
0
DPM
CYT w/o MoO4
CYT with MoO4
BSA
5 mM DTT
B
5
10
15
20
25

Figure A4. Effect of dithiothreitol on sedimentation coefficient of tick EcR. TE buffer with various DTT was used in this experiment. Curves were copied from figure A1 and A3. The large form was ~6S and the small form 3.4S.


600
500
400
300
200
100
0
DPM
B
5
10
15
20
25
30
fraction number
w/o DTT
0.5 mM DTT
5 mM DTT
BSA